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HUMAN COX-1 ALTERNATIVELY SPLICED VARIANTS AND METHODS OF  
USING SAME

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**HUMAN COX-1 ALTERNATIVELY SPLICED VARIANTS AND METHODS OF  
USING SAME**

**FIELD OF THE INVENTION**

This invention relates generally to molecular  
5 medicine and, more specifically, to alternatively spliced  
human cyclooxygenase-1 (COX-1).

**BACKGROUND INFORMATION**

Aspirin has been in use for over 100 years. The  
mechanism of action of this common drug was not fully  
10 appreciated until 1971 when it was discovered that the  
ability of aspirin to suppress inflammation lies primarily  
in its ability to inhibit the cyclooxygenase (COX) enzyme.  
Subsequently, two cyclooxygenase isoforms have been  
identified, designated as COX-1 and COX-2. The COX enzymes  
15 catalyze conversion of arachidonic acid to prostaglandin G<sub>2</sub>  
(PGG<sub>2</sub>) and prostaglandin H<sub>2</sub> (PGH<sub>2</sub>); (see Figure 1). PGH<sub>2</sub> is  
then converted to a variety of prostaglandins and other  
eicosanoids that play a role in inflammation and other  
disease processes. Aspirin inhibits the action of COX-1  
20 and COX-2 and thereby reduces prostaglandin and other  
eicosanoid levels and acts as an anti-inflammatory agent.

Prostaglandins (PG) are oxygenated fatty acids  
that bind to G-protein coupled receptors (GPCRs). Several  
25 naturally occurring prostaglandins, PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2α</sub>, and  
PGI<sub>2</sub>, have been identified. Prostaglandins produce numerous  
physiologic and pathophysiologic effects and regulate  
cellular processes in nearly every tissue. The wide  
spectrum of prostaglandin action includes effects on  
30 immune, endocrine, cardiovascular, renal and reproductive  
systems as well as the contraction and relaxation of smooth

muscle. Accordingly, drugs that effect prostaglandin production such as aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) have been used to prevent or alleviate a variety of conditions including, for  
5 example, cardiovascular disease, discomfort associated with minor injuries and headaches, and of severe pain caused by a variety of inflammatory and degenerative joint diseases.

Given the broad role of prostaglandins in normal human physiology, it is not surprising that systemic  
10 suppression of prostaglandin synthesis through inhibition of the COX enzymes can lead to unwanted side effects. In particular, individuals taking NSAIDs for even short periods of time can experience gastrointestinal and renal side effects, in addition to effects on other physiological  
15 systems. As many as 25% of individuals using NSAIDs experience some type of side effect, and as many as 5% develop serious health consequences such as gastric bleeding, ulceration, or perforation.

While COX-1 and COX-2 carry out essentially the  
20 same catalytic reaction and have similar primary protein structures, the expression patterns of these isoforms are distinct. In general, COX-1 is expressed constitutively in nearly all normal tissues, while COX-2 is expressed at low to undetectable levels in normal tissues but is induced in  
25 certain circumstances such as in response to injury or inflammation. Recently, drugs that selectively inhibit COX-2 (coxibs) have been proposed as a safer alternative to traditional NSAIDs. Although some of these selective  
inhibitors of COX-2 have demonstrated better  
30 gastrointestinal safety compared to traditional non-selective NSAIDs, questions remain regarding their effects on renal and cardiovascular systems. In addition, selective inhibitors of COX-2 do not appear to have the

protective cardiovascular effect observed with traditional NSAIDs such as aspirin.

A goal of clinical pharmacology and the pharmaceutical industry is the development of more selective drugs with greater efficacy and fewer side effects than those currently in use. In order to more effectively treat conditions where COX-1 modulators can be of benefit, such as inflammation and cardiovascular disease, COX-1 modulatory drugs with greater selectivity must be discovered. New COX-1 variants, such as alternatively spliced COX-1 polypeptides, can be more closely associated with a disease such as cardiovascular disease than the known COX-1 isoform and, thus, can be novel targets for drug discovery efforts, resulting in the discovery of drugs with greater efficacy or fewer side effects than drugs developed against the known COX-1 isoform.

Thus, there exists a need for the discovery of new COX-1 variants which can be used, for example, to design more specific drugs with fewer side effects. The present invention satisfies this need and provides related advantages as well.

#### SUMMARY OF THE INVENTION

The invention provides an isolated polypeptide containing the amino acid sequence of SEQ ID NO: 14, 16, 18, 20, 22 or 24. The invention also provides an isolated polypeptide containing a) an amino acid sequence having at least 50% amino acid identity with SEQ ID NO: 10, and b) an amino acid sequence selected from SEQ ID NOS: 14, 16, 18, 20, 22 or 24; or a conservative variant thereof. The invention further provides an isolated polypeptide containing the amino acid sequence of

SEQ ID NO: 2, 4, 6, or 8. The invention also provides a method for identifying a compound that modulates a COX-1 variant by contacting an isolated COX-1 variant or a COX-1 variant over-expressed in a genetically engineered cell with a compound and determining the level of an indicator which correlates with modulation of a COX-1 variant, where an alteration in the level of the indicator as compared to a control level indicates that the compound is a compound that modulates the COX-1 variant.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic diagram of the conversion of arachidonic acid to prostaglandins and other eicosanoids by the cyclooxygenase enzymes. The following abbreviations are used in Figure 1: NSAID: non-steroidal anti-inflammatory drug, PGG<sub>2</sub>: prostaglandin G<sub>2</sub>, PGH<sub>2</sub>: prostaglandin H<sub>2</sub>, PGF<sub>2</sub>α: prostaglandin F<sub>2</sub> alpha, PGD<sub>2</sub>: prostaglandin D<sub>2</sub>, PGE<sub>2</sub>: prostaglandin E<sub>2</sub>, PGI<sub>2</sub>: prostaglandin I<sub>2</sub>, TXA<sub>2</sub>: thromboxane A<sub>2</sub>.

Figure 2 shows the nucleotide sequence of human COX-1 variant ALT-1 (SEQ ID NO: 1). The underlined sequence indicates a nucleotide sequence which is not present in the known wild-type COX-1 enzyme (SEQ ID NO: 9). The start and stop codons for COX-1 variant ALT-1 are indicated in bolded capital letters.

Figure 3 shows the nucleotide sequence of human COX-1 variant ALT-2 (SEQ ID NO: 3). The underlined sequence indicates a nucleotide sequence which is not present in known wild-type COX-1 enzyme (SEQ ID NO: 4). The start and stop codons for COX-1 variant ALT-2 are indicated in bolded capital letters.

Figure 4 shows the nucleotide sequence of human COX-1 variant ALT-3 (SEQ ID NO: 5). The underlined sequence indicates a nucleotide sequence which is not present in the known wild-type COX-1 enzyme (SEQ ID NO: 9). The start and stop codons for COX-1 variant ALT-3 are indicated in bolded capital letters.

Figure 5 shows the nucleotide sequence of human COX-1 variant ALT-4 (SEQ ID NO: 7). The underlined sequence indicates a nucleotide sequence which is not present in the known wild-type COX-1 enzyme (SEQ ID NO: 9). The start and stop codons for COX-1 variant ALT-4 are indicated in bolded capital letters. The italicized letters indicate a novel nucleotide sequence derived from Exon B as shown in Figure 7.

Figure 6 shows a comparison of the amino acid sequences of the known wild-type human COX-1 enzyme (SEQ ID NO: 10), abbreviated as COX-1 WT, with human COX-1 variants ALT-1 (SEQ ID NO: 2), ALT-2 (SEQ ID NO: 4), ALT-3 (SEQ ID NO: 6), and ALT-4 (SEQ ID NO: 8). The first arrow in the amino terminal area of the polypeptides indicates the location where the wild-type human COX-1 enzyme and the COX-1 variants ALT-1, ALT-3 and ALT-4 begin to coincide and the second arrow indicates the location where the wild-type human COX-1 enzyme and COX-1 variant ALT-2 begin to co-incide.

Figure 7 shows the intron/exon structure of the human COX-1 genomic DNA clone AF440204. The figure schematically indicates which exons are found in COX-1 variants ALT-1 through ALT-4. Arg-120 is located at the opening of the hydrophobic fatty acid binding channel and is the counterion for the carboxylate group of arachidonate. His-388 and His-207 are the proximal and

distal heme ligands, respectively. Tyr-385 neighbors the heme group and bound arachidonate and is likely the residue that is converted to a tyrosyl radical and abstracts the (13-pro-S)-hydrogen from arachidonate, thereby initiating cyclooxygenase catalysis. Ser-530 is the site of aspirin acetylation.

Figure 8 shows distribution of mRNA from COX-1 variants ALT-1 through ALT-3 in various tissues using a reverse transcriptase-polymerase chain reaction RT-PCR procedure. The location of PCR products of the correct size for COX-1 variants ALT-1 through ALT-3 is indicated by an arrow. Sequences of primers used for PCR analysis are shown.

Figure 9 shows distribution of mRNA from COX-1 variant ALT-4 in various tissues using an RT-PCR procedure. The expression of COX-1 variant ALT-4 mRNA in the neuronal cell line SK-N-SH (SK control) and in SK-N-SH cells cultured with 20% fetal bovine serum (SK 20% FBS) is also shown. The location of a PCR product of the correct size for COX-1 variant ALT-4 is indicated by an arrow. Sequences of primers used for PCR analysis are shown.

Figure 10 shows induction of COX-1 variants ALT-1 and ALT-4 mRNA expression in SK-N-SH cells in response to culturing cells with 20% fetal bovine serum (FBS) for varying number of hours. C = control. The primers used in this experiment are identical to those listed in Figures 8 and 9.

### DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the exciting discovery of several novel COX-1 variants. Such COX-1 variants can be used to determine and refine the specificity of compounds that bind and modulate the known wild-type COX-1 enzyme. These COX-1 variants also can be used to identify a compound that differentially modulates or binds to a first COX-1 variant in relation to a second COX-1 variant, wild-type COX-1 enzyme, or wild-type COX-2 enzyme. Such a compound can be, for example, a compound that specifically binds to a novel COX-1 variant described herein.

As disclosed herein in Example I, several novel COX-1 variants were identified using the reverse transcription polymerase chain reaction (RT-PCR) and the following COX-1 primers: GGTTCCTGCTGTTCTGCTC (SEQ ID NO: 11) and TCACACTGGTAGCGGTCAAG (SEQ ID NO: 12). In particular, four novel alternatively spliced human COX-1 variants, designated herein COX-1 variant ALT-1, COX-1 variant ALT-2, COX-1 variant ALT-3, and COX-1 variant ALT-4, were identified as distinct from the wild-type human COX-1 enzyme (see Figures 2-6).

As further disclosed herein, sequence analysis of nucleic acid molecules encoding the alternatively spliced COX-1 variants revealed novel amino-terminal amino acid sequences. The amino acid sequences of the wild-type human COX-1 enzyme and the alternatively spliced COX-1 variants are shown in Figure 6. Comparison of the known wild-type human COX-1 enzyme amino acid sequence (SEQ ID NO: 10) to the alternatively spliced human COX-1 variants identified herein revealed the novel amino acid sequence MRKPRLM (SEQ ID NO: 14) at the

amino-terminus of COX-1 variants ALT-1 and ALT-3. As shown in Figure 7, the start site of COX-1 variants ALT-1 and ALT-3 start sites are located within a newly identified alternatively spliced exon (labeled "A" in Figure 7) which resides between exon 2 and exon 3 in the wild-type COX-1 enzyme sequence. The amino acid sequence at the junction of newly identified alternatively spliced exon A and exon 3 of the wild-type sequence is KPRLMNPCC (SEQ ID NO: 20), where the first five amino acids are encoded by the alternatively spliced exon A and the remaining four amino acids are residues derived from exon 3 (see Figures 6 and 7). As shown in Figures 6 and 7, the COX-1 variant ALT-3 differs from COX-1 variant ALT-1 by lacking part of exon 9.

As disclosed herein, the nucleotide sequence of COX-1 variant ALT-2 also contains alternatively spliced exon A (see Figure 7). In COX-1 variant ALT-2, exon A is spliced to exon 6 of the wild-type human COX-1 sequence whereas in COX-1 variants ALT-1 and COX-1 variant ALT-3, exon A is spliced to exon 3. The start site of COX-1 variant ALT-2 is located in exon A, resulting in the unique amino-terminal sequence MRKPRLR (SEQ ID NO: 18). The amino acid sequence at the junction of newly identified alternatively spliced exon A and exon 6 of the wild-type sequence is KPRLRKKQL (SEQ ID NO: 24), where the first five amino acids are encoded by the alternatively spliced exon A and the remaining four amino acids are derived from exon 6 (see Figures 6 and 7).

As further disclosed herein, the nucleotide sequence of COX-1 variant ALT-4 also contains alternatively spliced exon A. In addition, COX-1 variant ALT-4 contains a newly identified alternatively spliced exon (labeled "B" in Figure 7) which resides 3' to exon A

in COX-1 variant ALT-4 and 5' to exon 3 in the wild-type COX-1 enzyme sequence. The start site of COX-1 variant ALT-4 is located in exon A. The inclusion of exon B in COX-1 variant ALT-4 results in an amino-terminal sequence  
5 of MRKPRLSRCHDSPSSQNTWWAQDLNSV (SEQ ID NO: 16). The amino acid sequence at the junction between newly identified alternatively spliced exon B and exon 3 of the wild-type sequence is DLNSVNPCC (SEQ ID NO: 22), where the first five amino acids are encoded by the  
10 alternatively spliced exon B and the remaining four amino acids are residues derived from exon 3 (see Figures 6 and 7).

As further disclosed herein, expression of  
15 alternatively spliced human COX-1 variants ALT-1 through ALT-3 can be found in a variety of tissues including liver, kidney, brain, small intestine, spleen, lung, skeletal muscle, and heart (see Figure 8 and Example III). In particular, COX-1 variant ALT-1 mRNA  
20 was expressed in all of the tissue types examined. COX-1 variant ALT-2 was expressed at various levels in the tissues examined, with low to undetectable levels found in skeletal muscle. COX-1 variant ALT-3 also was expressed at various levels in the tissues examined, with  
25 low to undetectable levels found in liver, lung, skeletal muscle and heart.

As shown in Figure 9, human COX-1 variant ALT-4 mRNA was expressed at various levels in different tissues, with low to undetectable levels found in liver,  
30 brain, small intestine, skeletal muscle and heart. In addition, COX-1 variant ALT-4 mRNA was present in low to undetectable levels in the neuronal cell line SK-N-SH, but was induced by 20% fetal bovine serum treatment of these cells (see Figures 9 and 10). Induction of COX-1

variant ALT-4 mRNA in SK-N-SH cells was observed by three hours post induction (see Figure 10). In addition, as shown in Figure 10, COX-1 variant ALT-1 mRNA is increased in response to 20% fetal bovine serum treatment of  
5 SK-N-SH cells.

Based on these discoveries, the present invention provides novel alternatively spliced COX-1 variants and screening methods that rely on these  
10 variants. In particular, the invention provides an isolated polypeptide containing an amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22 or 24, which represent the unique amino-terminal and junctional portions of newly identified COX-1 variants ALT-1, ALT-2, ALT-3 and ALT-4.  
15 The present invention further provides an isolated polypeptide containing an amino acid sequence having at least 50% amino acid identity with SEQ ID NO: 10 and also containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof.  
20 Also provided herein is an isolated polypeptide containing the amino acid sequence of SEQ ID NO: 2, 4, 6 or 8. Further provided herein is an isolated polypeptide consisting of the amino acid sequence of SEQ ID NO: 2, 4, 6 or 8; or a conservative variant thereof. In one  
25 embodiment, the invention provides an isolated polypeptide consisting of the amino acid sequence of SEQ ID NO: 2, 4, 6 or 8.

The present invention further provides a method for identifying a compound that modulates a COX-1 variant  
30 by contacting an isolated COX-1 variant or a COX-1 variant over-expressed in a genetically engineered cell with a compound and determining the level of an indicator which correlates with modulation of the COX-1 variant, where an alteration in the level of the indicator as

compared to a control level indicates that the compound is a compound that modulates the COX-1 variant. The present invention also provides a method for identifying a compound that specifically binds to a COX-1 variant by  
5 contacting an isolated COX-1 variant or a COX-1 variant over-expressed in a genetically engineered cell with a compound and determining specific binding of the compound to the COX-1 variant.

The invention additionally provides a method  
10 for identifying a compound that differentially modulates a COX-1 variant by a) contacting an isolated COX-1 variant or a COX-1 variant over-expressed in a genetically engineered cell with a compound; b) determining the level of an indicator which correlates  
15 with modulation of the COX-1 variant; c) contacting a second COX enzyme with the compound; d) determining the level of a corresponding indicator which correlates with modulation of the second COX enzyme; and e) comparing the level of the indicator from step (b) with the level of  
20 the corresponding indicator from step (d), where a different level of the indicator from step (b) compared to the level of the corresponding indicator from step (d) indicates that the compound is a compound that differentially modulates the COX-1 variant.

25 Further provided herein is a method for identifying a compound that differentially binds to a COX-1 variant by a) contacting an isolated COX-1 variant or a COX-1 variant over-expressed in a genetically engineered cell with a compound; b) determining specific  
30 binding of the compound to the COX-1 variant; c) contacting a second COX enzyme with the compound; d) determining specific binding of the compound to the second COX enzyme; and e) comparing the level of specific

binding from step (b) with the level of specific binding from step (d), where a different level of specific binding from step (b) compared to the level of specific binding from step (d) indicates that the compound is a  
5 compound that differentially binds to the COX-1 variant.

The methods of the invention can be useful for designing drugs that bind to or modulate a wild-type COX-1 such as human COX-1 (SEQ ID NO: 10) in preference to one or more of the disclosed alternatively spliced  
10 COX-1 variants, or for identifying compounds that bind to or modulate one or more COX-1 variants in preference to other known COX-1 variants or either or both of the wild-type COX-1 or COX-2 enzymes. Compounds identified by a method of the invention can be therapeutically  
15 useful in preventing or reducing the severity of any of a variety of a conditions where modulation of the COX-1 enzyme or a COX-1 variant is beneficial.

As discussed above, the present invention relates to novel, alternatively spliced COX-1 variants.  
20 The COX-1 enzyme is a membrane-bound, heme-containing, homodimer of two 70-kD polypeptides that catalyzes the first two steps in prostaglandin, thromboxane and prostacyclin synthesis (Needleman et al., Annu. Rev. Biochem. 55:69-102 (1986)). Specifically, COX-1  
25 catalyzes two separate reactions; the first being the addition of molecular oxygen to arachidonic acid to form PGG (cyclooxygenase reaction), and the second being the further conversion of PGG<sub>2</sub> to the more stable PGH<sub>2</sub> by a peroxidase reaction (Yokoyama and Tanabe, Biochem.  
30 Biophys. Res. Commun. 165:888-894 (1989); and Hla, Prostaglandins 51:81-85 (1996)). The peroxidase activity of COX-1 catalyzes oxidation of a broad range of substrates. Inhibition of COX-1 cyclooxygenase activity

is responsible for decreased levels of prostaglandins and eicosinoids.

The COX-1 enzyme, also called prostaglandin H synthase (PGHS), prostaglandin G/H synthase (PTGS1), or  
5 prostaglandin-endoperoxide synthase 1, was first cloned  
by Needleman's group (Merlie et al., J. Biol. Chem.  
263:3550-3553 (1988)) and DeWitt and Smith (DeWitt and  
Smith, Proc. Natl. Acad. Sci. USA 85:1412-1416 (1988)).  
COX-1 has also been cloned from mouse (DeWitt et al., J.  
10 Biol. Chem. 265:5192-2198 (1990)), human (Funk et al.,  
FASEB J. 5:2304-2312 (1991)), avian (Xie et al., Proc.  
Natl. Acad. Sci. USA 88:2692-2696 (1991)) and rat (Feng  
et al., Arch. Biochem. Biophys. 307:361-368 (1993)).

15 An alternatively spliced form of the human  
COX-1 gene lacking part of exon 9 has been reported  
(Diaz, J. Biol. Chem. 267:10616-10822 (1992)). This form  
of COX-1 has been designated PTGS1 transcript variant 2  
or COX-1 variant 2 (GenBank Accession No. NM\_080591).  
20 COX-1 variant 2 contains the same amino-terminal and  
carboxy-terminal sequences as the wild-type human COX-1  
enzyme (SEQ ID NO: 10; GenBank Accession No. NM\_000962)  
but lacks the same part of exon 9 that is missing in  
COX-1 variant ALT-3 (SEQ ID NO: 6) disclosed herein. As  
25 described above, COX-1 variant ALT-3 contains a unique  
amino-terminal region (SEQ ID NO: 14) as compared to the  
wild-type human COX-1 enzyme (SEQ ID NO: 10) and known  
COX-1 variant 2.

Alternatively spliced forms of the COX-1 gene  
30 have also been identified in various species. For  
example, a form of the rat COX-1 lacking codons 1-36 has  
been described (Kitzler et al., Arch. Biochem. and  
Biophys. 316:856-863 (1995); Vogliagis et al., Am. J.

Physiol. Gastrointest. Liver Physiol. 278:G820-G827

(2000); Vogliagis et al., Carcinogenesis 22:869-874

(2001)). In addition, three alternatively spliced forms of the canine COX-1 gene have been described

5 (Chandrasekharan et al., Proc. Natl. Acad. Sci. USA

21:13926-13931 (2002)). Two of the canine variants

retain intron 1 of the COX-1 gene while the third variant does not contain intron 1 and lacks exons 5-8

(Chandrasekharan et al., *supra*, 2002).

10 COX-1 has been found in nearly all tissues under basal conditions (Crofford, J. Rheum. 24:15-19). Consistent with this broad pattern of expression pattern, one function of COX-1 is to provide prostaglandin precursors for homeostatic regulation in a variety of

15 tissues. A second function of COX-1 is to provide precursors for thromboxane synthesis in blood platelets, (Schafer, J. Clin. Pharmacol. 35:209-219). Platelets, which do not have nuclei, cannot produce an inducible enzyme in response to activating conditions. In the

20 presence of an NSAID such as aspirin, platelets are prevented from generating thromboxane during activation and fail to complete successful aggregation, inhibiting their thrombogenic potential. In the adjacent vascular endothelium, prostaglandins and eicosanoids play a

25 different role. The release of eicosanoids by activated platelets may provide both a substrate and stimulus for the generation of prostacyclin (PGI<sub>2</sub>) by the endothelium. This compound stimulates vasodilatation, counteracting the vasoconstrictor, thromboxane. Prostacyclin formation

30 is a function of COX-2 (Hennan et al., Circulation 104:820-825 (2001)).

Because prostacyclin antagonizes the platelet aggregation mediated by thromboxane, selective COX-2

inhibitors can suppress prostacyclin formation without concomitant inhibition of thromboxane, thereby resulting in an increased risk of thrombosis. As an example, a significant increase in thrombotic cardiovascular events was noted in humans treated with rofecoxib, a selective COX-2 inhibitor, in comparison with patients receiving naproxen, a non-selective NSAID (Fitzgerald et al., Clin. Exp. Rheumatol. 19 (Suppl 25):S31-S36 (2001)).

COX-1 also functions in other physiological systems where it can lead to vasodilatation in the presence of contractile conditions. For example, in both the kidney and the stomach, normal physiological stimuli are associated with dramatic changes in blood flow. During times of lowered blood volume, the kidney releases angiotensin and other factors to maintain blood pressure by systemic vasoconstriction (Palmer and Henrich, Semin. Nephrol. 15:214-227 (1995)). At the same time, angiotensin provokes prostaglandin synthesis in the kidney. COX-1 is expressed in the vasculature, glomeruli, and collecting ducts of the kidney, where it is involved in producing vasodilating prostaglandins, which maintain renal plasma flow and glomerular filtration rate during conditions of systemic vasoconstriction. In the presence of NSAIDs, this protective response can fail, leading to renal ischemia and damage in susceptible individuals (Zambraski, Semin. Nephrol. 15:205-213 (1995)). Similarly, in the gastric antrum, NSAID use can lead to ischemia and ultimately mucosal damage and ulceration (Trevethick et al., Gen. Pharmacol. 26:1455-1459 (1995)). The enzyme blocked by NSAIDs is thought to be COX-1 (Wallace, Gastroenterology 112:1000-1016 (1997)) that produces prostaglandins, which alter blood flow in the microcirculation of the gastric mucosa.

COX-1 also may play a role in the human eye, where the iris is the major site for producing prostaglandins. In the eye, prostaglandins regulate smooth muscle contraction, blood-aqueous barrier penetration and intraocular pressure (Matsuo and Cynader, Br. J. Ophthalmol. 77:110-114 (1993)). Using immunoprecipitation, constitutive COX-1 expression was detected in human iris homogenates, whereas COX-2 was only detected after stimulation with lipopolysaccharide (Van Haeringen et al., J. Ocul. Pharmacol Ther. 16:353-361 (2000)). Furthermore, the NSAID S(+)-flurbiprofen inhibits COX-1 70-fold more potently in human iris than in human blood (Haeringen et al., *supra*, 2000). Such a difference could be due, for example, to an alternatively spliced form of COX-1 in human iris or in whole blood. Such alternatively spliced forms of COX-1 can include an isolated polypeptide of the invention, for example, one of the COX-1 variants ALT-1, ALT-2, ALT-3, or ALT-4 disclosed herein.

20

The invention provides novel COX-1 variants which are alternatively spliced forms of the wild-type COX-1 enzyme. In one embodiment, the invention provides an isolated polypeptide containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22 or 24. In another embodiment, the invention provides an isolated polypeptide containing an amino acid sequence having at least 50% amino acid identity with SEQ ID NO: 10 and further containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof. In a further embodiment, the invention provides an isolated polypeptide containing the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8. Further provided herein is an isolated polypeptide consisting of the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8, or a

35

conservative variant thereof. In one embodiment, the invention provides an isolated polypeptide consisting of the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8.

The invention further provides a COX-1 variant  
5 binding agent which binds the amino acid sequence of SEQ ID NOS: 14, 16 or 18; or an epitope thereof. Such a COX-1 variant binding agent can be, without limitation, an antibody or antigen binding fragment thereof. The invention additionally provides a cell that includes an  
10 exogenously expressed polypeptide containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22 or 24; a cell which includes an exogenously expressed polypeptide containing an amino acid sequence having at least 50% amino acid identity with SEQ ID NO: 10 and further  
15 containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof; and a cell which includes an exogenously expressed polypeptide containing the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8. Further provided herein is an  
20 isolated polypeptide consisting of the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8 or a conservative variant thereof. In one embodiment, the invention provides an isolated polypeptide consisting of the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8.

25 The present invention also provides a method for identifying a compound that modulates a COX-1 variant by contacting an isolated COX-1 variant or a COX-1 variant over-expressed in a genetically engineered cell with a compound and determining the level of an indicator  
30 which correlates with modulation of a COX-1 variant, where an alteration in the level of the indicator as compared to a control level indicates that the compound is a compound that modulates the COX-1 variant. The

alteration can be, for example, an increase or decrease in the level of an indicator such as, without limitation, a prostaglandin such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). A method of the invention can be practiced with any of a variety of COX-1 variants such as an isolated polypeptide containing an amino acid sequence having at least 50% amino acid identity with SEQ ID NO: 10 and further containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof; an isolated polypeptide containing the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8; or an isolated polypeptide consisting of the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8. A method of the invention also can be practiced using any of a variety of COX-1 variants over-expressed or exogenously expressed in a genetically engineered cell. In one embodiment, the COX-1 variant is exogenously over-expressed in the genetically engineered cell. A variety of compounds can be screened according to the methods of the invention including, but not limited to, small molecules and polypeptides.

The present invention further provides a method for identifying a compound that specifically binds to a COX-1 variant by contacting an isolated COX-1 variant or a COX-1 variant over-expressed in a genetically engineered cell with a compound and determining specific binding of the compound to the COX-1 variant. In particular embodiments, a method of the invention is practiced using an isolated COX-1 variant such as a polypeptide containing an amino acid sequence having at least 50% amino acid identity with SEQ ID NO: 10 and further containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof; an isolated COX-1 variant containing the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8; or an isolated

COX-1 variant consisting of the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8. In another embodiment, a method of the invention is practiced using a COX-1 variant over-expressed or exogenously expressed in a genetically engineered cell, for example, a COX-1 variant exogenously over-expressed in a genetically engineered cell. In the methods of the invention, contacting can occur *in vivo* or *in vitro*, and the compounds to be screened can include, without limitation, small molecules and polypeptides.

The invention further provides a method for identifying a compound that differentially modulates a COX-1 variant by a) contacting an isolated COX-1 variant or a COX-1 variant over-expressed in a genetically engineered cell with a compound; b) determining the level of an indicator which correlates with modulation of a COX-1 variant; c) contacting a second COX enzyme with the compound; d) determining the level of a corresponding indicator which correlates with modulation of the second COX enzyme; and e) comparing the level of the indicator from step (b) with the level of the corresponding indicator from step (d), where a different level of the indicator from step (b) compared to the level of the corresponding indicator from step (d) indicates that the compound is a compound that differentially modulates the COX-1 variant. The COX enzyme can be, for example, a distinct COX-1 variant or a wild-type COX-1 or COX-2 from the same or a different species, or a functional fragment thereof. The human COX-2 nucleotide sequence (SEQ ID NO: 25) and amino acid sequence (SEQ ID NO: 26) can be found in GenBank at Accession No. NM\_000963. The level of the indicator from step (b) can be greater or less than the level of the indicator from step (d) and the indicator can be, for example, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>).

In particular embodiments, a method of the invention is practiced using an isolated COX-1 variant containing an amino acid sequence having at least 50% amino acid identity with SEQ ID NO: 10 and further containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof; an isolated COX-1 variant containing the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8; or an isolated COX-1 variant consisting of the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8. In another embodiment, a method of the invention is practiced using a COX-1 variant over-expressed in a genetically engineered cell, for example, a COX-1 variant exogenously over-expressed or exogenously expressed in a genetically engineered cell. In the methods of the invention, the compounds to be screened can include, without limitation, small molecules and polypeptides.

The invention further provides a method for identifying a compound that differentially binds to a COX-1 variant by a) contacting an isolated COX-1 variant or a COX-1 variant over-expressed in a genetically engineered cell with a compound; b) determining specific binding of the compound to the COX-1 variant; c) contacting a second COX enzyme with the compound; d) determining specific binding of the compound to the second COX enzyme; and e) comparing the level of specific binding from step (b) with the level of specific binding from step (d), where a different level of specific binding from step (b) compared to the level of specific binding from step (d) indicates that the compound is a compound that differentially binds to the COX-1 variant. The second COX enzyme can be, for example, a distinct COX-1 variant or a wild-type COX-1 or COX-2 from the same or a different species, or a functional fragment thereof.

The different level of specific binding can be an increased or decreased level of specific binding. In particular embodiments, a method of the invention is practiced using an isolated COX-1 variant containing an amino acid sequence having at least 50% amino acid identity with SEQ ID NO: 10 and further containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof; an isolated COX-1 variant containing the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8; or an isolated COX-1 variant consisting of the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8. In another embodiment, a method of the invention is practiced using a COX-1 variant over-expressed in a genetically engineered cell, for example, a COX-1 variant exogenously over-expressed in a genetically engineered cell. In the methods of the invention, contacting can occur *in vivo* or *in vitro*. One skilled in the art understands that the compounds to be screened include, yet are not limited to, small molecules and polypeptides.

The invention also provides an isolated nucleic acid molecule having a nucleotide sequence that encodes a polypeptide containing an amino acid sequence having at least 50% amino acid identity with SEQ ID NO: 10 and further containing the amino acid sequence of SEQ ID NOS: 20, 22 or 24; or a conservative variant thereof. The invention further provides an isolated nucleic acid molecule containing a nucleotide sequence that encodes the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8. In addition, the invention provides an isolated nucleic acid molecule consisting of a nucleotide sequence of SEQ ID NOS: 1, 3, 5 or 7. The invention further provides a vector containing a nucleic acid molecule having a nucleotide sequence that encodes a polypeptide containing an amino acid sequence having at least 50% amino acid

identity with SEQ ID NO: 10 and further containing the amino acid sequence of SEQ ID NOS: 20, 22 or 24; or a conservative variant thereof; a nucleic acid molecule containing a nucleotide sequence that encodes the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8; or a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NOS: 1, 3, 5 or 7. Host cells containing such a vector are further provided herein.

10                   From the foregoing, it is clear that the present invention relates, in part, to the identification of novel COX-1 variants. As used herein, the term "COX-1 variant" means a polypeptide containing an amino acid sequence that has at least 30% amino acid identity with  
15 the wild-type human COX-1 enzyme SEQ ID NO: 10 and further containing the amino acid sequence of (SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof. As non-limiting examples, a COX-1 variant can contain an amino acid sequence having, for example, at  
20 least 30% amino acid identity, at least 40% amino acid identity, at least 50% amino acid identity, at least 60% amino acid identity, at least 70% amino acid identity, at least 80% amino acid identity, at least 90% amino acid identity, or at least 95% amino acid identity with the  
25 amino acid sequence of the wild-type human COX-1 enzyme (SEQ ID NO: 10). As a non-limiting example, a COX-1 variant can contain an amino acid sequence having at least 50% amino acid identity with SEQ ID NOS: 10 and further containing the amino acid sequence of SEQ ID NOS:  
30 14, 16, 18, 20, 22, or 24; or a conservative variant thereof.

Based on the above, it is understood that species homologs of COX-1 variants that contain the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24;

or a conservative variant thereof, are encompassed by the definition of COX-1 variant as used herein. As non-limiting examples, an isolated polypeptide containing the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8, or  
5 consisting of the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8 or a conservative variant thereof is a COX-1 variant of the invention.

A COX-1 variant differs from the known  
10 wild-type human COX-1 polypeptide by containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof. As used herein in reference to a specified amino acid sequence such as SEQ ID NOS: 14, 16, 18, 20, 22 or 24, a "conservative  
15 variant" is a sequence in which one or more first amino acids are replaced by another amino acid or amino acid analog having at least one biochemical property similar to that of the first amino acid; similar properties include, yet are not limited to, similar size, charge,  
20 hydrophobicity or hydrogen-bonding capacity.

As an example, a conservative variant can be a sequence in which a first uncharged polar amino acid is conservatively substituted with a second (non-identical) uncharged polar amino acid such as cysteine, serine,  
25 threonine, tyrosine, glycine, glutamine or asparagine or an analog thereof. A conservative variant also can be a sequence in which a first basic amino acid is conservatively substituted with a second basic amino acid such as arginine, lysine, histidine, 5-hydroxylysine,  
30 N-methyllysine or an analog thereof. Similarly, a conservative variant can be a sequence in which a first hydrophobic amino acid is conservatively substituted with a second hydrophobic amino acid such as alanine, valine, leucine, isoleucine, proline, methionine, phenylalanine

or tryptophan or an analog thereof. In the same way, a conservative variant can be a sequence in which a first acidic amino acid is conservatively substituted with a second acidic amino acid such as aspartic acid or glutamic acid or an analog thereof; a sequence in which an aromatic amino acid such as phenylalanine is conservatively substituted with a second aromatic amino acid or amino acid analog, for example, tyrosine; or a sequence in which a first relatively small amino acid such as alanine is substituted with a second relatively small amino acid or amino acid analog such as glycine or valine or an analog thereof. It is understood that a conservative variant of SEQ ID NOS: 14, 16, 18, 20, 22 or 24 can have one, two, three, four, five, six, seven, eight or more conservative amino acid substitutions relative to the specified sequence and that such a conservative variant can include naturally and non-naturally occurring amino acid analogs.

It is also understood that a fragment of a COX-1 variant containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof, can be useful in a method of the invention. As non-limiting examples, a functional fragment of a COX-1 variant such as a heme-binding fragment or a fragment of a COX-1 variant that is involved in aspirin acetylation, such as a fragment containing Ser 530, can be useful in a method of the invention in place of the full-length COX-1 variant. Other fragments useful in the invention include, without limitation, fragments containing Arg 120, which is important for fatty acid and arylalkanoic acid COX inhibitors, and fragments containing Tyr 385, which forms the tyrosyl radical that initiates substrate oxygenation. As further understood by one skilled in the art, a COX-1

variant can optionally include non-homologous amino acid sequence. As non-limiting examples, a COX-1 variant can contain an epitope tag or can be fused to a non-homologous polypeptide such as glutathione S-transferase.

As discussed above, the COX-1 variants ALT-1, ALT-2, ALT-3, and ALT-4 include an amino acid sequence that is not present in the wild-type COX-1 enzyme SEQ ID NO: 10 (see Figure 6). For example, the alternatively spliced COX-1 variants ALT-1 and ALT-3 contain unique amino-terminal amino acid sequence disclosed herein as SEQ ID NO: 14, and the alternatively spliced COX-1 variant ALT-4 contains unique amino-terminal amino acid sequence disclosed herein as SEQ ID NO: 16 and the alternatively spliced COX-1 variant ALT-2 contains the unique amino-terminal amino acid sequences disclosed herein as SEQ ID NO: 18. Furthermore, a nine amino acid sequence spanning the junction between newly identified alternatively spliced exon A and exon 3 of the wild-type sequence is disclosed herein as KPRLMNPCC (SEQ ID NO: 20), where the first five amino acids are encoded by the alternatively spliced exon A and the remaining four amino acids are residues derived from exon 3 (see Figures 6 and 7). In addition, a nine amino acid sequence spanning the junction between newly identified alternatively spliced exon B and exon 3 is disclosed herein as DLNSVNPCC (SEQ ID NO: 22), where the first five amino acids are encoded by the alternatively spliced exon B and the remaining four amino acids are residues derived from exon 3 (see Figures 6 and 7). Further, a nine amino acid sequence spanning the invention between newly identified spliced exon A and exon 6 is disclosed herein as KPRLRKKL (SEQ ID NO: 24), where the first five amino acids are encoded by the alternatively spliced exon A and

the remaining four amino acids are residues derived from exon 6. Thus, the invention provides an isolated polypeptide containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22 or 24. The invention further  
5 provides an isolated polypeptide containing an amino acid sequence having at least 50% amino acid identity with SEQ ID NO: 10 and further containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof. As non-limiting examples,  
10 the invention provides an isolated polypeptide containing the amino acid sequence of SEQ ID NOS: 2, 6 or 8, or an isolated polypeptide consisting of the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8.

Further provided herein is an isolated  
15 polypeptide containing substantially the same amino acid sequence as SEQ ID NOS: 2, 4, 6 or 8, or consisting of substantially the same amino acid sequence as SEQ ID NOS: 2, 4, 6 or 8. The term "substantially the same," when used herein in reference to an amino acid sequence,  
20 means a polypeptide having a similar, non-identical sequence that is considered by those skilled in the art to be a functionally equivalent amino acid sequence. An amino acid sequence that is substantially the same as a reference amino acid sequence can have at least 70%, at  
25 least 80%, at least 90%, or at least 95% or more identity to the reference sequence. The term substantially the same also includes amino acid sequences encompassing, for example, modified forms of naturally occurring amino acids such as D-stereoisomers, non-naturally occurring  
30 amino acids, amino acid analogs and mimetics, so long as the polypeptide containing such a sequence retains a functional activity of the reference COX-1 variant. A functional activity of a COX-1 variant of the invention can be, without limitation, the ability to convert

arachidonic acid to a prostaglandin such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), the ability to bind heme, or the ability to be acetylated by aspirin or inhibited by other NSAIDs.

5           It is understood that minor modifications in primary amino acid sequence can result in a polypeptide that has a substantially equivalent function as compared to a polypeptide of the invention. These modifications can be deliberate, as through site-directed mutagenesis, 10 or may be accidental, produced, for example, through spontaneous mutation. For example, it is understood that only a portion of the entire primary structure of a COX-1 variant can be required in order to, for example, convert arachidonic acid to PGE<sub>2</sub> or another prostaglandin. 15 Moreover, functional fragments of a COX-1 variant of the invention containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof, similarly are included within the definition of substantially the same amino acid sequence; as set forth 20 above, such functional fragments retain at least one biological function of the COX-1 variant. It is understood that various molecules can be attached to a COX-1 variant or other polypeptide of the invention. These molecules include, without limitation, heterologous 25 polypeptides or peptides, carbohydrates, lipids, liposomes, phage or chemical moieties such as radioactive or fluorescent labels.

          The invention further provides a COX-1 variant binding agent which binds the amino acid sequence of SEQ 30 ID NOS: 14, 16 or 18, or an epitope thereof. As discussed above, SEQ ID NO: 14 represents the unique amino-terminal amino acid sequence of alternatively spliced COX-1 variants ALT-1 and ALT-3, SEQ ID NO: 16 represents the unique amino-terminal amino acid sequence

of alternatively spliced COX-1 variant ALT-4 and SEQ ID NO: 18 represents the unique amino terminal amino acid sequences of alternatively spliced COX-1 variant ALT-2. A COX-1 variant binding agent of the invention can be, 5 without limitation, an antibody or antigen binding fragment thereof which binds the amino acid sequence of SEQ ID NOS: 14, 16 or 18; or an epitope thereof.

As used herein, the term "COX-1 variant binding agent" means a molecule, such as a simple or complex 10 organic molecule, carbohydrate, peptide, peptidomimetic, protein, glycoprotein, lipoprotein, lipid, nucleic acid molecule, antibody, aptamer or the like that specifically binds, competitively or non-competitively, the unique COX-1 variant amino-terminal amino acid sequence 15 disclosed herein as SEQ ID NOS: 14, 16 or 18; or an epitope thereof. It is understood that such a binding agent does not specifically bind to a wild-type COX-1 such as SEQ ID NO: 10 since a wild-type COX-1 does not contain the unique amino-terminal amino acid sequence 20 disclosed herein as SEQ ID NOS: 14, 16 or 18.

A COX-1 variant binding agent of the invention can be a polypeptide that specifically binds with high affinity or avidity to SEQ ID NOS: 14, 16 or 18, without substantial cross-reactivity to other unrelated 25 sequences. The affinity of a COX-1 variant binding agent of the invention generally is greater than about  $10^4 \text{ M}^{-1}$  and can be greater than about  $10^6 \text{ M}^{-1}$ . A COX-1 variant binding agent of the invention also can bind with high affinity such as an affinity greater than  $10^4 \text{ M}^{-1}$  30 to  $10^{10} \text{ M}^{-1}$ . Specific examples of binding agents of the invention include, but are not limited to, polyclonal and monoclonal antibodies that specifically bind an epitope within SEQ ID NOS: 14, 16 or 18; and nucleic acid

molecules, nucleic acid analogs, and small organic molecules, identified, for example, by affinity screening of a nucleic acid or small molecule library against SEQ ID NOS: 14, 16 or 18, or an epitope thereof. For certain applications, a COX-1 variant binding agent that preferentially recognizes a particular conformational or post-translationally modified state of SEQ ID NOS: 14, 16 or 18 can be preferred. It is understood that a COX-1 variant binding agent of the invention can be labeled with a detectable moiety, if desired, or rendered detectable by specific binding to a detectable secondary agent.

In one embodiment, a COX-1 variant binding agent of the invention is an antibody or antigen-binding fragment thereof. As used herein, the term "antibody" is used in its broadest sense to mean a polyclonal or monoclonal antibody or an antigen-binding fragment of such an antibody. Such an antibody of the invention is characterized by having specific binding activity for SEQ ID NOS: 14, 16 or 18, or an epitope thereof, of at least about  $1 \times 10^5 \text{ M}^{-1}$ . Thus, Fab,  $\text{F(ab')}_2$ , Fd and Fv fragments of an antibody, which retain specific binding activity for SEQ ID NOS: 14, 16 or 18, or an epitope thereof, are included within the definition of antibody as used herein. Specific binding activity can be readily determined by one skilled in the art, for example, by comparing the binding activity of the antibody to SEQ ID NOS: 14, 16 or 18, versus a control sequence. Methods of preparing polyclonal or monoclonal antibodies are well known to those skilled in the art. See, for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1988).

It is understood that the term antibody includes naturally occurring antibodies as well as non-naturally occurring antibodies such as, without limitation, single chain antibodies, chimeric, 5 bi-functional and humanized antibodies, and antigen-binding fragments thereof. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, produced recombinantly or obtained, for example, by screening combinatorial libraries 10 consisting of variable heavy chains and variable light chains as described in Huse et al., Science 246:1275-1281 (1989). These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bi-functional antibodies are well known to those skilled 15 in the art (Winter and Harris, Immunol. Today 14:243-246 (1993); Ward et al., Nature 341:544-546 (1989); Harlow and Lane, *supra*, 1988; Hilyard et al., Protein Engineering: A practical approach (IRL Press 1992); and Borrabeck, Antibody Engineering, 2d ed. (Oxford 20 University Press 1995)).

An antibody of the invention can be prepared using as an antigen a polypeptide or peptide containing SEQ ID NOS: 14, 16 or 18, or an epitope thereof, which can be prepared, for example, from natural sources, 25 produced recombinantly, or chemically synthesized. Such a polypeptide or peptide is a functional antigen if the polypeptide or peptide can be used to generate an antibody that specifically binds SEQ ID NOS: 14, 16 or 18, or an epitope thereof. As is well known in the art, 30 a non-antigenic or weakly antigenic polypeptide or peptide can be made antigenic by coupling the polypeptide or peptide to a carrier molecule such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various other carrier molecules and methods for coupling

a polypeptide or peptide to a carrier molecule are well known in the art (see, for example, Harlow and Lane, *supra*, 1988). An antigenic polypeptide or peptide can also be generated by expressing the polypeptide or  
5 peptide as a fusion protein, for example, fused to glutathione S transferase, polyHis or the like. Methods for expressing polypeptide fusions are well known to those skilled in the art as described, for example, in Ausubel et al., Current Protocols in Molecular Biology  
10 (Supplement 47), John Wiley & Sons, New York (1999).

The present invention also provides a cell that includes an exogenously expressed polypeptide containing the amino acid sequence of SEQ ID NO: 14, 16, 18, 20, 22 or 24. Further provided herein is a cell that includes  
15 an exogenously expressed polypeptide containing an amino acid sequence having at least 50% amino acid identity with SEQ ID NO: 10 and further containing the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof. The invention provides,  
20 for example, a cell that includes an exogenously expressed polypeptide containing the amino acid sequence of SEQ ID NO: 2, 4, 6, or 8, or consisting of the amino acid sequence of SEQ ID NO: 2, 4, 6 or 8.

A cell of the invention can be generated by  
25 expressing a nucleic acid molecule encoding the polypeptide to be exogenously expressed in a suitable host cell, such as, without limitation, a bacterial cell, yeast cell, insect cell, oocyte or other amphibian cell, or mammalian cell, using methods well known in the art.  
30 Suitable expression vectors are well known in the art and include vectors in which a nucleic acid molecule is operatively linked to a regulatory element such as a promoter or enhancer region that is capable of regulating

expression of a linked nucleic acid molecule.  
Appropriate expression vectors include, without  
limitation, those that can be replicated in eukaryotic or  
prokaryotic cells, those that remain episomal as well as  
5 those which integrate into the host cell genome, and  
those including constitutive, inducible or regulated  
promoters, enhancers or other regulatory elements.

Suitable expression vectors for prokaryotic or  
eukaryotic cells are well known to those skilled in the  
10 art (see, for example, Ausubel et al., *supra*, 1999).  
Eukaryotic expression vectors can contain, for example, a  
regulatory element such as, but not limited to, the SV40  
early promoter, the cytomegalovirus (CMV) promoter, the  
mouse mammary tumor virus (MMTV) steroid-inducible  
15 promoter, the Moloney murine leukemia virus (MMLV)  
promoter, and the like. One skilled in the art will know  
or can readily determine an appropriate expression vector  
for a particular host cell.

Useful expression vectors optionally contain a  
20 regulatory element that provides cell or tissue specific  
expression or inducible expression of the operatively  
linked nucleic acid molecule. One skilled in the art can  
readily determine an appropriate tissue-specific promoter  
or enhancer that allows expression of a polypeptide of  
25 the invention in a desired tissue. Furthermore, any of a  
variety of inducible promoters or enhancers can also be  
included in an expression vector for regulated expression  
of a polypeptide of the invention. Such inducible  
systems include, yet are not limited to, a tetracycline  
30 inducible gene regulatory region (Gossen & Bijard, Proc.  
Natl. Acad. Sci. USA, 89:5547-5551 (1992); Gossen et al.,  
Science, 268:1766-1769 (1995); Clontech, Palo Alto, CA);  
a metallothionein promoter inducible by heavy metals; an

insect steroid hormone responsive gene regulatory region responsive to ecdysone or related steroids such as muristerone (No et al., Proc. Natl. Acad. Sci. USA, 93:3346-3351 (1996); Yao et al., Nature, 366:476-479 (1993); Invitrogen, Carlsbad, CA); a mouse mammary tumor virus (MMTV) gene regulatory region induced by steroids such as glucocorticoid and estrogen (Lee et al., Nature, 294:228-232 (1981); and a heat shock promoter.

An expression vector useful in the invention can be a viral vector such as, without limitation, a retrovirus, adenovirus, adeno-associated virus, lentivirus, or herpesvirus vector. Viral based systems provide the advantage of being able to introduce relatively high levels of a heterologous nucleic acid molecule into a variety of cells. Additionally, certain viral vectors can introduce heterologous DNA into non-dividing cells. A variety of suitable viral expression vectors are well known in the art and include, without limitation, herpes simplex virus vectors (U.S. Patent No. 5,501,979), vaccinia virus vectors (U.S. Patent No. 5,506,138), cytomegalovirus vectors (U.S. Patent No. 5,561,063), modified Moloney murine leukemia virus vectors (U.S. Patent No. 5,693,508), adenovirus vectors (U.S. Patent Nos. 5,700,470 and 5,731,172), adeno-associated virus vectors (U.S. Patent No. 5,604,090), constitutive and regulatable retrovirus vectors (U.S. Patent Nos. 4,405,712; 4,650,764 and 5,739,018, respectively), papilloma virus vectors (U.S. Patent Nos. 5,674,703 and 5,719,054), and the like.

A cell of the invention transiently or stably expresses the exogenously expressed polypeptide. Expression vectors for transient or stable expression of a polypeptide can be introduced into cells using

transfection methods well known to one skilled in the art. Such methods include, without limitation, infection using viral vectors, lipofection, electroporation, particle bombardment and transfection such as

5 calcium-phosphate mediated transfection. Detailed procedures for these methods can be found in Sambrook et al., Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press (1989), and the references cited therein. Useful mammalian expression vectors and methods

10 of introducing such vectors into mammalian cells either ex vivo or in vivo are well known in the art. As non-limiting examples, a plasmid expression vector can be introduced into a cell by calcium-phosphate mediated transfection, DEAE dextran-mediated transfection,

15 lipofection, polybrene- or polylysine-mediated transfection, electroporation, or by conjugation to an antibody, gramicidin S, artificial viral envelope or other intracellular carrier. A viral expression vector can be introduced into a cell by infection or

20 transduction, for example, or by encapsulation in a liposome. It further is understood that polypeptides can be delivered directly into cells using a lipid-mediated delivery system (Zelphati et al., J. Biol. Chem. 276:35103-35110 (2001)) to produce a cell that contains

25 exogenously expressed COX-1 variant.

Exemplary host cells that can be used to exogenously express a polypeptide of the invention include, yet are not limited to, mammalian primary cells; established mammalian cell lines such as COS, CHO, HeLa,

30 NIH3T3, HEK 293, and HEK 293/EBNA cells; amphibian cells such as *Xenopus* embryos and oocytes; and other vertebrate and invertebrate cells. Exemplary host cells further include, without limitation, insect cells such as *Drosophila* and *Spodoptera frugiperda*, including Sf9

cells, Sf21 cells and other cells compatible with baculovirus expression systems (Murakimi et al., Cytokine, 13(1):18-24, (2001)); yeast cells such as *Saccharomyces cerevisiae*, *Saccharomyces pombe*, or *Pichia pastoris*; and prokaryotic cells such as *Escherichia coli*. Following transfection, cells exogenously expressing a polypeptide of the invention can be selected, for example, using drug resistance. A quantitative assay such as, for example, immunoblot analysis, immunoprecipitation or ELISA can determine the amount of a polypeptide of the invention expressed in a transfected cell. Such methods are known to one skilled in the art and can be found, for example, in Ausubel et al., *supra*, 1989, or in Harlow et al., *supra*, 1988.

Further provided herein are methods for identifying a compound that modulates a COX-1 variant, identifying a compound that differentially modulates a COX-1 variant, identifying a compound that specifically binds a COX-1 variant, and identifying a compound that differentially binds to a COX-1 variant. In particular, the invention provides a method for identifying a compound that modulates a COX-1 variant by contacting a COX-1 variant with a compound and determining the level of an indicator which correlates with modulation of a COX-1 variant, where an alteration in the level of the indicator as compared to a control level indicates that the compound is a compound that modulates the COX-1 variant. Further provided herein are methods for identifying a compound that modulates a COX-1 variant by contacting an isolated COX-1 variant or a COX-1 variant over-expressed or exogenously expressed in a genetically engineered cell with a compound and determining the level of an indicator which correlates with modulation of a COX-1 variant, where an alteration in the level of the

indicator as compared to a control level indicates that the compound is a compound that modulates the COX-1 variant.

As used herein in reference to a COX-1 variant, the term "modulates" means the ability to alter a characteristic of a COX-1 variant. A characteristic of a COX-1 variant that can be altered can include, without limitation, an amount, activity, or physical conformation of a COX-1 variant. As a non-limiting example, a compound that modulates a COX-1 variant can increase or decrease the binding of a COX-1 variant to a compound like aspirin or other NSAID. As a further non-limiting example, a compound can increase or decrease the binding of a COX-1 variant to an intracellular signaling molecule that initiates a signal transduction pathway within a cell. As still further non-limiting examples, a compound that modulates a COX-1 variant can increase or decrease an activity of a COX-1 variant such as, without limitation, cyclooxygenase activity or peroxidase activity. It is understood that compounds that modulate a COX-1 variant include compounds that specifically bind to a COX-1 variant as well as compounds that do not specifically bind to a COX-1 variant.

A method of the invention for identifying a compound that modulates a COX-1 variant involves determining the level of an indicator which correlates with modulation of the COX-1 variant, where an alteration in the level of the indicator as compared to a control level indicates that the compound modulates the COX-1 variant. As used herein, the term "indicator" means a detectable substance which is altered qualitatively or quantitatively in response to modulation of a COX-1 variant. An indicator can be a substance that is

normally present in a cell, for example, an element such as oxygen, a prostaglandin, eicosinoid, or signal transduction molecule, or a substance that is exogenously expressed or otherwise added to a cell, the level of which correlates with modulation of a COX-1 variant. Examples include, without limitation, luciferase and chemiluminescent substrates. Signal transduction molecules are intracellular substances such as, without limitation, cyclic AMP, inositol phosphates and calcium, the level of which can be altered in response to modulation of a COX-1 variant.

As understood by those of skill in the art, assay methods for identifying compounds that modulate a COX-1 variant generally require comparison to a control. For example, in a method of the invention an alteration in the level of an indicator which correlates with modulation of a COX-1 variant is compared to a control level of the indicator. One type of a control is a sample that is treated substantially the same as the COX-1 variant which is contacted with a compound, with the distinction that the control sample is not exposed to the compound. Controls include, but are not limited to, historical reference values and samples that are assayed simultaneously or sequentially in comparison to the COX-1 variant which is contacted with a compound.

In one embodiment, a method of the invention is practiced using a prostaglandin such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) as the indicator. In another embodiment, a method of the invention is practiced using a chemiluminescent substrate for COX-1 activity, for example, the chemiluminescent substrate for COX-1 peroxidative activity available from Assay Designs, Inc. (Ann Arbor, MI) or Stressgen Biotechnologies (Victoria, British

Columbia). Exogenously expressed substances such as, without limitation, luciferase, b-galactosidase and green fluorescent protein (GFP) also can be indicators useful in a method of the invention.

5 Further provided herein are methods for identifying a compound that specifically binds to a COX-1 variant by contacting a COX-1 variant with a compound and determining specific binding of the compound to the COX-1 variant. Additionally provided herein are methods for  
10 identifying a compound that specifically binds to a COX-1 variant by contacting an isolated COX-1 variant or a COX-1 variant over-expressed or exogenously expressed in a genetically engineered cell with a compound and determining specific binding of the compound to the COX-1  
15 variant.

As used herein in reference to a compound and a COX-1 variant, the term "specific binding" means binding with an affinity for the target COX-1 variant that is measurably higher than the affinity for an unrelated  
20 polypeptide such as an unrelated enzyme. For example, a polypeptide or small molecule compound that specifically binds a COX-1 variant has an affinity for the COX-1 variant that is measurably higher than its affinity for an unrelated enzyme. Binding affinity can be low or high  
25 affinity so long as the binding is sufficient to be detectable. For example, a compound can specifically bind a COX-1 variant with a binding affinity (Kd) of about  $10^{-4}$  M or less,  $10^{-5}$  M or less,  $10^{-6}$  M or less,  $10^{-7}$  M or less,  $10^{-8}$  M or less, or  $10^{-9}$  M or less. In  
30 addition, specific binding includes both covalent and non-covalent binding. For example, several enzymes bind to their substrates and catalyze reactions without covalently binding the substrate molecule. Several

methods for detecting or measuring specific binding are well known in the art and discussed further below.

The screening methods of the invention can be practiced using, for example, a COX-1 variant  
5 over-expressed or exogenously expressed in a genetically engineered cell. As used herein, the term "genetically engineered cell" means a cell having genetic material which is altered by the hand of man. Such a cell can contain a transient or permanent alteration of its  
10 genetic material including, for example, an alteration in genomic or episomal genetic material. The genetic material in a genetically engineered cell can be altered using, without limitation, an exogenously expressed nucleic acid molecule, chemical mutagen or transposable  
15 element. It is understood that a genetically engineered cell can contain one or multiple man-made alterations, for example, a cell can be co-transfected with more than one expression vector. As used herein in relation to a COX-1 variant in a genetically engineered cell, the term  
20 "over-expressed" means having a protein level of a COX-1 variant greater than the level seen in a corresponding non-genetically engineered cell.

A COX-1 variant can be over-expressed in a genetically engineered cell, for example, by exogenously  
25 expressing a nucleic acid molecule encoding the COX-1 variant in a cell as described herein above. It is understood that a COX-1 variant can be over-expressed in a cell that does not normally express the COX-1 variant, or in a cell that naturally expresses the endogenous  
30 COX-1 variant. As a non-limiting examples, a COX-1 variant can be over-expressed in a cell that expresses the same or a different endogenous COX-1 variant at a low level. In addition, a COX-1 variant can be

over-expressed in a genetically engineered cell, for example, by expressing a regulatory molecule in the cell to increase expression of the endogenous COX-1 variant. Another example of a method whereby a COX-1 variant can  
5 be over-expressed in a genetically engineered cell is recombination of a heterologous regulatory region such as, without limitation, a promoter, enhancer or 3' regulator, in the cell such that the heterologous regulatory region results in over-expression of  
10 endogenous COX-1 variant. As understood by one skilled in the art, over-expression of a COX-1 variant in a genetically engineered cell includes, without limitation, over-expression of the variant on the surface of the cell, within a cell membrane or in the cytosolic portion  
15 of the cell.

A COX-1 variant also can be over-expressed in a cell using a chemical agent. Thus, the invention provides a method for identifying a compound that  
20 modulates a COX-1 variant by contacting the COX-1 variant with a compound, where the COX-1 variant is over-expressed in a cell using a chemical agent, and determining the level of an indicator which correlates with modulation of the COX-1 variant, where an alteration  
25 in the level of the indicator as compared to a control level indicates that the compound is a compound that modulates the COX-1 variant. The invention also provides a method for identifying a compound that specifically binds to a COX-1 variant by contacting the COX-1 variant  
30 with a compound, where the COX-1 variant is over-expressed in a cell using a chemical agent, and determining specific binding of the compound to the COX-1 variant. Chemical agents that result in over-expression of a COX-1 variant include, without limitation, chemicals  
35 that induce the level or activity of regulatory factor,

such as a transcription factor, that is involved in COX-1 variant expression.

As disclosed above, the methods of the invention can be practiced with a cell that over-expresses a COX-1 variant. In addition, it is understood that an extract of a cell that over-expresses a COX-1 variant such as a genetically engineered cell that over-expresses a COX-1 variant can be useful in the methods of the invention. Methods for generating different types of cellular extracts including, without limitation, whole cell extracts, fractionated extracts, membrane extracts, cytosolic extracts and nuclear extracts are well known in the art. As a non-limiting example, COX-1 variant enriched plasma membrane fractions can be obtained by continuous or discontinuous gradients of, for example, sucrose.

An isolated COX-1 variant also can be useful in a screening method of the invention. As used herein in reference to a COX-1 variant, the term "isolated" means the COX-1 variant is substantially separated from other polypeptides. For example, an isolated COX-1 variant derived from a cell can be substantially purified away from other polypeptides in the cell. Furthermore, an isolated COX-1 variant can be a single subunit of the COX-1 variant or can be a homodimer similar to the structure of the known wild-type human COX-1 enzyme. An isolated COX-1 variant also can contain fused heterologous sequences or other associated non-polypeptide components; for example, an isolated COX-1 variant can be associated with a natural or artificial lipid containing membrane. In one embodiment, a method of the invention is practiced with an isolated COX-1 variant that contains an amino acid sequence having

at least 50% amino acid identity with SEQ ID NO: 10 and further contains the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20, 22, or 24; or a conservative variant thereof. In another embodiment, a method of the  
5 invention is practiced with an isolated COX-1 variant that contains the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8, or that consists of the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8.

10 A COX-1 variant can be prepared in isolated form using conventional biochemical purification methods, starting either from tissues containing the desired COX-1 variant or from recombinant sources. A COX-1 variant can be isolated by any of a variety of methods well-known in  
15 the art, including, but not limited to, precipitation, gel filtration, ion-exchange, reverse-phase and affinity chromatography, and combinations thereof. Other well-known methods for protein isolation are described in Deutscher et al., Guide to Protein Purification: Methods  
20 in Enzymology Vol. 182, (Academic Press, (1990)). Methods suitable for isolation of a COX-1 variant of the invention using biochemical purification are known in the art as described for example, in Rowlinson et al., J. Biol. Chem. 274:23305-23310 (1999), and Marnett et al.,  
25 Mol. Pharm. 26:328-335 (1984). Purification of the COX-1 variant can be routinely monitored, for example, by an immunological assay or functional assay such as a cyclooxygenase or peroxidase assay.

An isolated COX-1 variant also can be produced  
30 by chemical synthesis. As a non-limiting example, synthetic isolated COX-1 variants, including fragments thereof, can be produced using an Applied Biosystems, Inc. Model 430A or 431A automatic peptide synthesizer (Foster City, CA) employing the chemistry provided by the

manufacturer. Methods for synthesizing isolated polypeptides are well known in the art (see, for example, Bodanzsky, Principles of Peptide Synthesis (1st ed. & 2d rev. ed.), Springer-Verlag, New York, New York (1984 & 5 1993), see Chapter 7; Stewart and Young, Solid Phase Peptide Synthesis, (2d ed.), Pierce Chemical Co., Rockford, Illinois (1984)).

In the methods of the invention for identifying a compound that modulates, or specifically binds to, a  
10 COX-1 variant, an isolated COX-1 variant or COX-1 variant over-expressed or exogenously expressed in a genetically engineered cell can be contacted with a compound in a solution under conditions suitable for interaction between the COX-1 variant and compound. Such contact can  
15 occur *in vitro*, such as in an isolated cell in cell culture, in a whole or partially purified cell extract, or with an isolated polypeptide. As used herein, the term "*in vitro*" means in an artificial environment outside of a living organism or cell. Contacting  
20 performed in a test tube, microcentrifuge tube, 96 well plate, 384 well plate, 1536 well plate or other assay format outside of an organism and without using living cells occurs *in vitro*. Contact performed in cells or tissues that have been fixed and are therefore dead  
25 (sometimes referred to as *in situ* experiments) or using cell-free extracts from cells occurs *in vitro*. Contact can also occur *in vivo* using, for example, whole animals.

Conditions suitable for contacting an isolated  
30 COX-1 variant or COX-1 variant over-expressed or exogenously expressed in a genetically engineered cell with a compound are dependent on the characteristics of the COX-1 variant and the compound. For example, the overall charge of the COX-1 variant and the compound can

be considered when adjusting the salt concentration or pH of a buffering solution to optimize the specific binding or modulation of the COX-1 variant by the compound. Usually a salt concentration and pH in the physiological  
5 range, for example, about 100 mM KCl and pH 7.0 are reasonable starting points. In addition, other components such as glycerol or protease inhibitors can be added to the solution, for example, to inhibit polypeptide degradation. It is understood that the  
10 stability of the contact between the COX-1 variant and the compound can be effected by the salt concentration and temperature at which such contact occurs and that the optimal salt concentration and temperature for contact can be routinely determined by those skilled in the art.  
15 For example, reactions can be performed on ice (4°C), at room temperature (about 25°C) or at body temperature (37°C). Suitable conditions can be similar or identical to conditions used for binding of a compound to the wild-type human COX-1. Such conditions are known in the  
20 art and include, for example, preincubating cells that express the COX-1 variant with compound for 30 minutes at 25°C, then adding arachidonic acid at a final concentration of 5 or 30 mM for an additional 10 minute incubation at 37°C, as described in Chadrsekharan et al.,  
25 *supra*, 2002 (see also Example II). Assayed can then be performed for cyclooxygenase (COX) activity, for example, by radioimmunoassay for PGE<sub>2</sub>.

The screening methods of the invention are useful for identifying compounds that modulate or  
30 differentially modulate, or that specifically bind or differentially bind a COX-1 variant. As used herein, the term "compound" means a molecule of natural or synthetic origin. A compound can be, without limitation, a small organic or inorganic molecule, polypeptide, peptide,

peptidomimetic, non-peptidyl compound, carbohydrate, lipid, antibody or antibody fragment, aptamer, or nucleic acid molecule. In one embodiment, the compound is a small organic molecule. It is understood that a compound  
5 can have a known or unknown structure, and can be assayed as an isolated molecule or as part of a population of compounds such as in a pool or as a portion of a library.

As is understood by one skilled in the art, a compound can specifically bind to a COX-1 variant without  
10 modulating the COX-1 variant; specifically bind to a COX-1 variant, thereby modulating the COX-1 variant; or modulate a COX-1 variant without specifically binding the COX-1 variant. Compounds that specifically bind to a COX-1 variant can include, without limitation,  
15 arachidonic acid or other fatty acids, and aspirin or other NSAIDs; such molecules can be identical or similar to, or structurally distinct from those that specifically bind the wild-type COX-1 isoform. A compound that modulates a COX-1 variant but does not directly bind to  
20 the COX-1 variant can be, for example, a compound that binds to or effects the activity of a polypeptide in a cell, where that polypeptide increases or decreases the level of a COX-1 variant. Such compounds include, without limitation, transcription or translation  
25 regulatory factors, signal transduction polypeptides; kinases and phosphatases; and anti-sense oligonucleotides, inhibitor RNA molecules and ribozymes, that act on the nucleic acid that encodes the COX-1 variant; and molecules that affect the expression or  
30 activity of COX-1 inhibitors.

Compounds that modulate or specifically bind to a COX-1 variant further include, but are not limited to, agonists and antagonists for other proteins. Agonists

and antagonists for proteins such as receptors are well known in the art.

A library of compounds can be useful in the screening methods of the invention. Such a library can be a random collection of compounds or a focused collection of compounds, for example, compounds that are rationally designed or pre-selected based on one or more physical or functional characteristics. For example, a library of compounds related to aspirin or one or more other compounds can be useful in the screening methods of the invention. A variety of NSAID compounds are known in the art and include, without limitation, aspirin, flurbiprofen, ketoprofen, etodolac, ibuprofen, piroxicam, carprofen, celecoxib, diclofenac, flunixin, meloxicam, deracoxib, NS-398, DUP-697, and SC-58125.

Libraries useful in the methods of the invention include, yet are not limited to, natural product libraries derived from, without limitation, microorganisms, animals, plants, and marine organisms; combinatorial chemical or other chemical libraries such as those containing randomly synthesized compounds; combinatorial libraries containing structural analogs of NSAIDs or other known compounds, or random or biased assortments of, for example, small organic molecules, polypeptides, oligonucleotides, and combinations thereof. Still other libraries of interest include peptidomimetic, multiparallel synthetic collections, and recombinatorial libraries. Combinatorial and other chemical libraries are known in the art, as described, for example, in Myers, Curr. Opin. Biotechnol. 8:701-707 (1997). Appropriate libraries can be assembled from catalog sources such as Cayman Chemical Co. (Ann Arbor, MI), BIOMOL Research Laboratories, Inc. (Plymouth Meeting,

PA), Tocris Cooksoon Inc. (Ellisville, MO), and others. In addition to NSAID-related compounds, these libraries can include, without limitation, fatty acids, fatty acid amides and esters, and eicosanoids.

5

In a screening method of the invention, the members of a library of compounds can be assayed for activity individually, in pools, or *en masse*. An example of *en masse* screening to identify a compound that  
10 modulates or specifically binds to a COX-1 variant is as follows: a library of compounds is assayed in pools for the ability to modulate or specifically bind a COX-1 variant; the sub-population which modulates or specifically binds the COX-1 variant is subdivided; and  
15 the assay is repeated as needed in order to isolate an individual compound or compounds from the library that modulate or specifically bind the COX-1 variant.

The methods of the invention can utilize high  
20 throughput screening (HTS) techniques to identify compounds that modulate or specifically bind to a COX-1 variant. HTS assays permit screening of large numbers of compounds in an efficient manner. Cell-based high throughput screening systems include, but are not limited  
25 to, yeast-based assay systems and mammalian cell expression systems (Jayawickreme and Kost, Curr. Opin. Biotechnol. 8:629-634 (1997)). Automated and miniaturized high throughput screening assays are also useful in the methods of the invention (Houston and  
30 Banks, Curr. Opin. Biotechnol. 8:734-740 (1997)). High throughput screening assays are designed to identify "hits" or "lead compounds" having the desired modulating or specific binding activity, from which modified compounds can be prepared to improve a property of the  
35 initial lead compound. Chemical modification of the

"hit" or "lead compound" can be based on an identifiable structure/activity relationship (SAR) between the "hit" and a COX-1 variant of the invention. It is understood that assays such as the conversion of arachidonic acid to  
5 a prostaglandin such as PGE<sub>2</sub> and other assays of cyclooxygenase or peroxidase activity can be performed as conventional or high through-put screening assays to identify a compound that modulates or specifically binds to a COX-1 variant according to a method of the  
10 invention. In addition, as understood by one skilled in the art, a radioligand binding or other assay can be modified, for example, by using two COX-1 inhibitors that bind COX-1 in a competition assay.

15 Various types of assays can be useful for identifying a compound that modulates or specifically binds to a COX-1 variant in a method of the invention. For example, several assays can be used to measure specific binding of a compound to a COX-1 variant in a  
20 method of the invention. An assay that can be used for measuring specific binding of a compound to a COX enzyme is a radioligand binding assay. Radioligand binding assays can be performed on cells or in solution, for example, using isolated cell membranes. As a  
25 non-limiting example, cells or cell membranes that transiently or stably over-express a COX-1 variant can be incubated with a ligand including a novel or known ligand such as a radioactively labeled NSAID. After washing away any unbound radioactively labeled NSAID, compounds  
30 of interest can be incubated with the cells. After incubation, the solution around the cells is collected and the amount of radioactively labeled NSAID in the solution is determined using, for example, a scintillation counter. Compounds that specifically bind  
35 to the COX-1 variant displace the radioactively labeled

NSAID from the COX enzyme and thereby increase radioactively labeled NSAID in solution. As understood by one skilled in the art, a ligand such as a NSAID also can be labeled with a non-radioactive moiety such as a  
5 fluorescent moiety.

A variety of other assays well known in the art can be used to determine specific binding of a compound to a COX-1 variant in a method of the invention. Such methods for determining specific binding to a COX-1  
10 variant include, without limitation, detecting specific binding of a labeled compound to a COX-1 variant which is immobilized. For example, a compound can be conjugated to a radiolabel, fluorescent label or enzyme label such as alkaline phosphatase, horse radish peroxidase or  
15 luciferase. Labeled compound can then bind to a COX-1 variant, for example a COX-1 variant membrane preparation, which is immobilized, for example, on a solid support such as a latex bead. Unbound compound is washed away, and the amount of specifically bound  
20 compound can be detected based on its label. Fluorescently labeled compound can also be bound to a COX-1 variant in solution and bound complexes detected, for example using a fluorescence polarization assay (Degterev et al., Nature Cell Biology 3:173-182 (2001)).  
25 Such assays also can be performed where the COX-1 variant is labeled and the compound is immobilized or in solution. One skilled in the art understands that a variety of additional means can be used to determine specific binding to a COX-1 variant; as non-limiting  
30 examples, specific binding of a compound to a <sup>15</sup>N-labeled COX-1 variant can be detected using nuclear magnetic resonance (NMR), or specific binding can be determined using an antibody that specifically recognizes a ligand-bound COX-1 variant. In addition, binding of a

compound to a COX-1 variant can be determined or confirmed using X-ray crystallography. Conditions for crystallography can be based on those used to solve the crystal structure of wild-type sheep seminal vesicle  
5 COX-1 (Picot et al, Nature 367:243-249 (1994)).

High-throughput assays for determining specific binding to a COX-1 variant further include, but are not limited to, scintillation proximity assays (Alouani, Methods Mol. Biol. 138:135-41 (2000)). Scintillation  
10 proximity assays involve the use of a fluomicrosphere coated with an acceptor molecule, such as an antibody, to which an antigen will bind selectively in a reversible manner. For example, a compound can be bound to a fluomicrosphere using an antibody that specifically binds  
15 to the compound, and contacted with a  $^3\text{H}$  or  $^{125}\text{I}$  labeled COX-1 variant. If the labeled COX-1 variant specifically binds to the compound, the radiation energy from the labeled COX-1 variant is absorbed by the fluomicrosphere, thereby producing light which is easily measured. Such  
20 assays can also be performed where the COX-1 variant is bound to the fluomicrosphere, and the compound is labeled.

Additional assays suitable for determining specific binding of a compound to a COX-1 variant in a  
25 screening method of the invention include, without limitation, UV or chemical cross-linking assays (Fancy, Curr. Opin. Chem. Biol. 4:28-33 (2000)) and biomolecular interaction analyses (Weinberger et al., Pharmacogenomics 1:395-416 (2000)). Specific binding of a compound to a  
30 COX-1 variant can be determined by cross-linking the compound and variant, if they are in contact with each other, using UV or a chemical cross-linking agent. In addition, a biomolecular interaction analysis (BIA) can

detect whether two components are in contact with each other. In such an assay, one component, such as a COX-1 variant, for example, an isolated COX-1 variant or a membrane preparation containing a COX-1 variant, is bound  
5 to a BIA chip, and a second component such as a compound is passed over the chip. If the two components specifically bind, the contact results in an electrical signal, which is readily detected.

In addition, virtual computational methods and  
10 the like can be used to identify compounds that modulate or specifically bind to a COX-1 variant in a screening method of the invention. Exemplary virtual computational methodology involves virtual docking of small-molecule compounds on a virtual representation of a COX-1 variant  
15 structure in order to determine or predict specific binding. See, for example, Shukur et al., *supra*, 1996; Lengauer et al., Current Opinions in Structural Biology 6:402-406 (1996); Choichet et al., Journal of Molecular Biology 221:327-346 (1991); Cherfils et al., Proteins  
20 11:271-280 (1991); Palma et al., Proteins 39:372-384 (2000); Eckert et al., Cell 99:103-115 (1999); Loo et al., Med. Res. Rev. 19:307-319 (1999); and Kramer et al., J. Biol. Chem. (2000).

Assays useful in the methods of the invention  
25 that do not directly measure binding to a COX-1 variant include, for example, assays that measure COX-1 enzyme activity. Such assays include, without limitation, measurement of uptake of oxygen using an oxygraph, measurement of the conversion of arachidonic acid to  
30 prostaglandins or eicosinoids, and measurement of a chemiluminescent substrate for COX-1 peroxidase activity. Prostaglandin or eicosinoid synthesis from arachidonic acid or other precursors can be measured using any

standard detection method known in the art such as, for example, radioimmunoassays (RIAs) or enzyme-linked immunosorbent assays (ELISAs). A prostaglandin synthesis assay is described in Example II as is an assay that  
5 measures the turn-over of a COX-1 substrate or co-substrate such as *N,N,N,N'*-tetramethyl-*p*-phenylenediamine (TMPD). In addition, an example of a chemiluminescent assay for COX-1 peroxidase activity such as available from Assay  
10 Designs, Inc. (Ann Arbor, MI) or Stressgen Biotechnologies (Victoria, British Columbia) is described in Example II.

In addition to the methods described above for identifying a compound that modulates or specifically  
15 binds a COX-1 variant, the invention also provides related methods for identifying a compound that differentially modulates or differentially binds to a COX-1 variant. It is understood that the COX-1 variants, cells, compounds, indicators, conditions for contacting,  
20 and assays described above also can be applied to methods for identifying a compound that differentially modulates or differentially binds to a COX-1 variant.

In particular, the invention provides a method for identifying a compound that differentially modulates  
25 a COX-1 variant by a) contacting an isolated COX-1 variant or a COX-1 variant over-expressed or exogenously expressed in a genetically engineered cell with a compound; b) determining the level of an indicator which correlates with modulation of a COX-1 variant; c)  
30 contacting a second COX enzyme with the compound; d) determining the level of a corresponding indicator which correlates with modulation of the second COX enzyme; and e) comparing the level of the indicator from step (b)

with the level of the corresponding indicator from step (d), where a different level of the indicator from step (b) compared to the level of the corresponding indicator from step (d) indicates that the compound is a compound  
5 that differentially modulates the COX-1 variant.

As described above, an indicator is a detectable substance which is altered qualitatively or quantitatively in response to modulation of a COX-1 variant. A "corresponding indicator" is an indicator  
10 that can be compared to the indicator which correlates with modulation of the COX-1 variant in step (b). For example, a corresponding indicator can be the same indicator as the indicator which correlates with modulation of the COX-1 variant in step (b). A  
15 corresponding indicator also can be a different indicator as the indicator which correlates with modulation of the COX-1 variant in step (b) so long as the corresponding indicator can be compared to the indicator which correlates with modulation of the COX-1 variant in step  
20 (b). As a non-limiting example, the indicator in step (b) can be prostaglandin  $E_2$  ( $PGE_2$ ), and the corresponding indicator can be a substance whose amount is directly correlated with prostaglandin  $E_2$  ( $PGE_2$ ) level, such as a breakdown product of  $PGE_2$ . As a further non-limiting  
25 example, the indicator in step (b) and corresponding indicator in step (d) can be related molecules, such as two different fluorophores. In one embodiment, the level of the indicator which correlates with modulation of the COX-1 variant in step (b) is greater than the level of  
30 the corresponding indicator from step (d). In another embodiment, the level of the indicator which correlates with modulation of the COX-1 variant in step (b) is less than the level of the corresponding indicator from step (d).

The invention also provides a method for identifying a compound that differentially binds to a COX-1 variant by a) contacting an isolated COX-1 variant or a COX-1 variant over-expressed or exogenously  
5 expressed in a genetically engineered cell with a compound; b) determining specific binding of the compound to the COX-1 variant; c) contacting a second COX enzyme with the compound; d) determining specific binding of the compound to the second COX enzyme; and e) comparing the  
10 level of specific binding from step (b) with the level of specific binding from step (d), where a different level of specific binding from step (b) compared to the level of specific binding from step (d) indicates that the compound is a compound that differentially binds to the  
15 COX-1 variant. In one embodiment, the different level of specific binding is an increased level of binding of the compound to the COX-1 variant as compared to the second COX enzyme. In another embodiment, the different level of specific binding is a decreased level of binding of  
20 the compound to the COX-1 variant as compared to the second COX enzyme.

As disclosed above in regard to other methods, a COX-1 variant useful in a method of the invention for identifying a compound that differentially modulates or  
25 differentially binds a COX-1 variant can be any of a variety of COX-1 variants such as an isolated polypeptide containing an amino acid sequence having at least 50% amino acid identity with SEQ ID NO: 10 and further having the amino acid sequence of SEQ ID NOS: 14, 16, 18, 20,  
30 22, or 24; or a conservative variant thereof; an isolated polypeptide containing the amino acid sequence of SEQ ID NO: 2, 4, 6 or 8; or an isolated polypeptide consisting of the amino acid sequence of SEQ ID NOS: 2, 4, 6 or 8. In addition, the COX-1 variant can be over-expressed or

exogenously expressed in a genetically engineered cell. For example, the COX-1 variant can be exogenously over-expressed in a genetically engineered cell.

In the methods of the invention for identifying  
5 a compound that differentially modulates or differentially binds a COX-1 variant, the second COX enzyme can be any COX enzyme of interest. For example, the second COX enzyme can be, without limitation, any other COX enzyme such as a different COX-1 variant, a  
10 wild-type COX-1, wild-type COX-2, a known variant of COX-1 or COX-2, or a functional fragment of these polypeptides. In one embodiment, the second COX enzyme contains the amino acid sequence SEQ ID NO: 10, or a functional fragment thereof. It is understood that the  
15 second COX enzyme can be, for example, expressed in a cell endogenously or exogenously or can be an isolated COX enzyme polypeptide.

In one embodiment, a method of the invention is practiced using a COX-1 variant and second COX enzyme  
20 which are expressed in different cells of the same or different cell type. In addition, the methods of the invention can be practiced using a COX-1 variant and second COX enzyme which are expressed in the same cell, for example, where the COX-1 variant does not have  
25 identical binding and signal transduction effects as the co-expressed second COX enzyme.

As understood by one skilled in the art, in the methods of the invention for identifying a compound that modulates, differentially modulates, specifically binds,  
30 or differentially binds a COX-1 variant the order of the steps can be changed. In addition, steps can be performed simultaneously or sequentially.

The invention further provides an isolated nucleic acid molecule having a nucleotide sequence that encodes a polypeptide containing an amino acid sequence  
5 having at least 50% amino acid identity with SEQ ID NO: 10 and further containing the amino acid sequence of SEQ ID NOS: 20, 22 or 24, or a conservative variant thereof. The invention also provides an isolated nucleic acid molecule containing a nucleotide sequence that  
10 encodes the amino acid sequence of SEQ ID NO: 2, 4, 6 or 8. The invention further provides an isolated nucleic acid molecule consisting of a nucleotide sequence of SEQ ID NO: 1, 3, 5 or 7.

An isolated nucleic acid molecule can be a DNA  
15 or RNA molecule and further can have a sense or complementary anti-sense strand or both. It is understood that an isolated nucleic acid molecule of the invention can be a double-stranded or a single-stranded molecule, an RNA or DNA molecule, and can optionally  
20 include non-coding sequence. DNA molecules of the invention include cDNA molecules as well as wholly or partially chemically synthesized DNA sequences.

The nucleic acid molecules of the invention optionally include heterologous nucleic acid sequences  
25 that are not part of the COX-1 variant-encoding sequence in nature. Such a heterologous nucleic acid sequence can be optionally separated from the COX-1 variant-encoding sequence by an encoded cleavage site that facilitates removal of non-COX-1 variant polypeptide sequences from  
30 the expressed fusion protein. Heterologous nucleic acid sequences include, without limitation, sequences encoding poly-histidine sequences, FLAG tags and other epitopes; glutathione-S-transferase, thioredoxin, and maltose

binding protein domains or other domains or sequences that facilitate purification or detection of a fusion protein containing a COX-1 variant of the invention.

An isolated nucleic acid molecule of the invention encoding SEQ ID NOS: 20, 22 or 24 has a nucleotide sequence that is distinct from the nucleotide sequence of the human COX-1 genomic clone AF440204 since the nucleotide sequence which encodes the amino acid sequence SEQ ID NOS: 20, 22 or 24 spans the junction between newly identified alternatively spliced exons and conserved exons 3 or 6. The intron/exon structure of the human COX-1 genomic clone AF440204 is shown in Figure 7 along with the intron/exon structure of COX-1 variants ALT-1, ALT-2, ALT-3 and ALT-4. As understood by one skilled in the art, an intron starts with the dinucleotide GT and ends with the dinucleotide AG.

The invention further provides a vector containing a nucleic acid molecule having a nucleotide sequence that encodes a polypeptide containing an amino acid sequence having at least 50% amino acid identity with SEQ ID NO: 10 and that includes the amino acid sequence of SEQ ID NOS: 20, 22 or 24, or a conservative variant thereof. The invention also provides a vector which includes a nucleic acid molecule containing a nucleotide sequence that encodes the amino acid sequence of SEQ ID NO: 2, 4, 6 or 8, or conservative variants thereof, or consisting of the amino acid sequence of SEQ ID NO: 2, 4, 6, or 8. In addition, the invention provides a vector containing the nucleotide sequence of SEQ ID NO: 1, 3, 5, or 7, or consisting of the nucleotide sequence of SEQ ID NO: 1, 3, 5 or 7. The invention

further provides a host cell including a vector which contains a nucleic acid molecule of the invention.

Vectors are useful, for example, for subcloning  
5 and amplifying a nucleic acid molecule encoding a polypeptide of the invention and for recombinantly expressing the encoded COX-1 variant or other polypeptide. Vectors of the invention include, without limitation, viral vectors such as bacteriophage,  
10 baculovirus and retrovirus vectors; cosmids and plasmids; and, particularly for cloning large nucleic acid molecules, bacterial artificial chromosome vectors (BACs) and yeast artificial chromosome vectors (YACs). Such vectors are commercially available, and their uses are  
15 well known in the art. Vectors further encompass expression vectors such as those discussed herein above.

The invention also provides an isolated nucleic acid molecule containing a nucleotide sequence that encodes the amino acid sequence of SEQ ID NOS: 20, 22 or  
20 24. Such a nucleic acid molecule of the invention can be used, without limitation, in recombinant cloning methods or as a nucleic acid probe. The amino acid sequence of SEQ ID NO: 20 or 22 contains nine amino acids which begin with five amino acid residues that correspond to the  
25 amino acid sequence present in newly identified exon A or B and end with four amino acid residues that correspond to the amino acid sequence in exon 3, an exon which is present in human wild-type COX-1 as well as the COX-1 variants ALT-1, ALT-3, and ALT-4. The amino acid  
30 sequence of SEQ ID NO: 24 contains nine amino acids which begin with five amino acid residues that correspond to the amino acid sequence present in newly identified exon A and end with four amino acid residues that correspond to the amino acid sequence in exon 6.

As non-limiting examples, nucleic acid molecules of the invention can be derived from the unique nucleotide sequence which surrounds the junction between newly identified alternatively spliced exon A and

5 conserved exon 3 present in COX-1 variants ALT-1 and ALT-3, and can include, for example, 20 nucleotides spanning the splice junction. The COX-1 variants ALT-1 and ALT-3 include the nucleic acid sequence

gcggacccaggggcgccac (SEQ ID NO: 27) at the 5' splice

10 junction and ccgaggctcatgaatccctg (SEQ ID NO: 28) at the 3' splice junction. The COX-1 variant ALT-2 also includes the nucleic acid sequence gcggacccaggggcgccac (SEQ ID NO: 27) at the 5' splice junction. This variant, in which exon A is spliced to exon 6, contains the

15 nucleic acid sequence ccgaggctcaggaagaagca (SEQ ID NO: 29) at the 3' splice junction.

In addition, nucleic acid molecules of the invention can be derived from the unique nucleotide sequence which surrounds the COX-1 variant ALT-4 junction

20 between newly identified alternatively spliced exon B and conserved exon 3. For example, nucleic acid molecules containing 20 nucleotides spanning the splice junction are as follows. The COX-1 variant ALT-4 includes the nucleic acid sequence gcggacccaggggcgccac (SEQ ID NO:

25 27) at the 5' splice junction, just as for COX-1 variants ALT-1, ALT-2 and ALT-3, but contains ctgaactcagtgaatccctg (SEQ ID NO: 30) at the 3' splice junction.

As is understood by one skilled in the art, a nucleic acid molecule of the invention can incorporate

30 nucleotide sequence in addition to the nucleotide sequence of SEQ ID NOS: 27, 28, 29 or 30. For example, a nucleic acid molecule of the invention can include further naturally occurring sequence at the 5' or 3' end

of SEQ ID NOS: 27, 28, 29 or 30. Also, for example, a nucleic acid molecule of the invention can include one or more additional heterologous sequences such as nucleotide sequences encoding restriction enzyme sites or epitope  
5 tags. As non-limiting examples, nucleic acid molecules of the invention can be used in hybridization reactions such as Southern and Northern blots, to encode polypeptide sequence in recombinant cloning methods, or as primers in polymerase chain reactions.

10 The invention also provides an isolated nucleic acid molecule having a nucleotide sequence that encodes a polypeptide containing or consisting of substantially the same amino acid sequence as SEQ ID NO: 2, 4, 6 or 8 as described above. In addition, the invention provides an  
15 isolated nucleic acid molecule containing or consisting of substantially the same nucleotide sequence as SEQ ID NO: 1, 3, 5 or 7.

The invention further provides a method for preventing or reducing the severity of a disease  
20 associated with COX-1 or a COX-1 variant in a subject by introducing into the subject a compound that modulates or differentially modulates a COX-1 variant or another compound identified by a method of the invention. The invention also provides a method for preventing or  
25 reducing the severity of a cardiovascular disorder in a subject by introducing into the subject a compound that modulates or differentially modulates a COX-1 variant or another compound identified by a method of the invention. In addition, the invention provides a method for  
30 preventing or reducing the severity of ocular hypertension in a subject by introducing into the subject a compound that modulates or differentially modulates a COX-1 variant or another compound identified by a method

of the invention. Such a compound can be used, without limitation, to prevent or reduce the severity of glaucoma.

As used herein, a "disease associated with  
5 COX-1 or a COX-1 variant" means any disease or condition  
in which modulation of the activity of the wild-type  
COX-1 enzyme or a COX-1 variant can be beneficial. It is  
understood that the underlying cause of the disease or  
condition may or may not be due to an abnormality in  
10 expression or activity of a wild-type COX-1 enzyme or a  
COX-1 variant.

A disease or condition associated with COX-1 or  
a COX-1 variant can be, without limitation, pain, fever,  
a cardiovascular disorder or an ocular disorder such as  
15 glaucoma, ocular hypertension, uveitis, allergic  
conjunctivitis and related disorders. Additional  
diseases or conditions associated with COX-1 or a COX-1  
variant can include, without limitation, diseases  
involving inflammation, for example, rheumatoid arthritis,  
20 ankylosing spondylitis, osteoarthritis, bursitis,  
tendinitis, and gout. As non-limiting examples, such a  
compound can be used in humans for prophylactic treatment  
of a cardiovascular disorder or to prevent or treat pain  
such as headache, muscle ache, or pain caused by any of a  
25 variety of inflammatory or degenerative joint diseases.

As discussed above, the iris is the major site  
in the human eye for production of prostaglandins, which  
30 regulate smooth muscle contraction, blood-aqueous barrier  
penetration and intraocular pressure (Matsuo and Cynader,  
*supra*, 1993). COX-1 appears to be constitutively  
expressed in the human iris, whereas COX-2 expression is

triggered by lipopolysaccharides (Van Haeringen et al.,  
*supra*, 2000). Furthermore, the NSAID S(+)-flurbiprofen  
inhibits COX-1 70-fold more potently in human iris than  
in human blood (Haeringen et al., *supra*, 2000), a  
5 difference which may be due to expression of an  
alternatively spliced form of COX-1 in human iris or  
whole blood.

Thus, a compound identified by the methods of  
10 the invention can be used, without limitation, to prevent  
or reduce the severity of uveitis, which is inflammation  
of the uvea or the middle layer of the eye. The role of  
prostaglandins in uveitis is established. The uvea  
consists of three structures: the iris, the ciliary body  
15 and the choroid. Inflammation occurring in any of these  
three structures is termed uveitis. Inflammation in  
uveitis may involve any, but not necessarily all, of  
these three structures. Depending upon which structures  
are inflamed, uveitis can be further subcategorized into  
20 one of three main diagnoses including: 1) iritis or  
anterior uveitis, 2) cyclitis or intermediate uveitis,  
and 3) choroiditis or posterior uveitis.

Furthermore, a compound that modulates or  
differentially modulates a COX-1 variant or which is  
25 otherwise identified by a method of the invention can be  
used alone or in combination with one or more different  
compounds or other therapeutic agents or procedures for  
treatment of uveitis. Agents that are currently used in  
the treatment of uveitis include, but are not limited to,  
30 steroids, mydriatics, and immunosuppressants such as  
cyclosporin, azathioprine, methotrexate, mycophenolate,  
mofetil (Cellcept), tacrolimus and anti-tumor necrosis  
factor (TNF).

Other ocular conditions that can be prevented or treated with a compound that modulates or differentially modulates a COX-1 variant by a method of the invention include, without limitation, diabetic  
5 retinopathy; macular edema such as that associated with diabetes; conditions of retinal degeneration such as glaucoma, macular degeneration such as age-related macular degeneration (ARMD) and retinitis pigmentosa; retinal dystrophies; inflammatory disorders of the  
10 retina; vascular occlusive conditions of the retina such as retinal vein occlusions or branch or central retinal artery occlusions; retinopathy of prematurity; retinopathy associated with blood disorders such as sickle cell anemia; elevated intraocular pressure; ocular  
15 itch; damage following retinal detachment; damage or insult due to vitrectomy, retinal or other surgery; and other retinal damage including therapeutic damage such as that resulting from laser treatment of the retina, for example, pan-retinal photocoagulation for diabetic  
20 retinopathy or photodynamic therapy of the retina, for example, for age-related macular degeneration. Ocular conditions that can be prevented or treated with a compound that modulates or differentially modulates a COX-1 variant by a method of the invention further  
25 include, without limitation, genetic and acquired optic neuropathies such as optic neuropathies characterized primarily by loss of central vision, for example, Leber's hereditary optic neuropathy (LHON), autosomal dominant optic atrophy (Kjer disease) and other optic neuropathies  
30 such as those involving mitochondrial defects, aberrant dynamin-related proteins or inappropriate apoptosis; and optic neuritis such as that associated with multiple sclerosis, retinal vein occlusions or photodynamic or laser therapy. See, for example, Carelli et al.,

Neurochem. Intl. 40:573-584 (2002); and Olichon et al.,  
J. Biol. Chem. 278:7743-7746 (2003).

A compound that modulates or differentially  
5 modulates a COX-1 variant or another compound identified  
by a method of the invention also can be useful for  
preventing or treating pain. The term pain, as used  
herein, includes, without limitation, inflammatory pain,  
headache pain, muscle pain, visceral pain, neuropathic  
10 pain, and referred pain. Pain can be continuous or  
intermittent, of short duration such as acute pain, or of  
long duration such as chronic pain. Chronic pain is  
distinguished from acute pain, which is immediate,  
generally high threshold, pain brought about by injury  
15 such as a cut, crush, burn, or by chemical stimulation  
such as that experienced upon exposure to capsaicin, the  
active ingredient in chili peppers.

The methods of the invention further can be  
used, without limitation, to treat chronic or other  
20 headache pain such as pain associated with cluster  
headaches, tension headaches or chronic daily headaches;  
muscle pain including, but not limited to, that  
associated with back or other spasm; inflammatory pain or  
other symptoms resulting, for example, from spondylitis  
25 or arthritis such as rheumatoid arthritis, gouty  
arthritis, or osteoarthritis; gout; bursitis; painful  
menstruation and fever. In addition, the methods of the  
invention can be used, for example, to treat pain  
associated with injury, surgery, dental procedures,  
30 dysmenorrhea, labor and other pain associated with the  
female reproductive system, and systemic illness such as,  
without limitation, cancer. It is understood that these  
and other conditions which may respond to NSAIDs can be  
prevented or treated using a compound that modulates or

differentially modulates a COX-1 variant disclosed herein.

As described above, a compound that modulates or differentially modulates a COX-1 variant or another  
5 compound identified by a method of the invention also can be useful for preventing or treating an immune disease, for example, without limitation, uveitis. In addition, such a compound can be used to prevent or treat a disease associated with allergy, such as, without limitation,  
10 allergic conjunctivitis.

A compound that modulates or differentially modulates a COX-1 variant or another compound identified by a method of the invention also can be useful for preventing or treating a cardiovascular disorder. Such  
15 cardiovascular diseases include, but are not limited to, atherosclerosis; thrombosis; restenosis; vasculitis including autoimmune and viral vasculitis such as polyarteritis nodosa, Churg-Strass syndrome, Takayasu's arteritis, Kawasaki Disease and Rickettsial vasculitis;  
20 atherosclerotic aneurisms; myocardial hypertrophy; congenital heart diseases (CHD); ischemic heart disease and anginas; acquired valvular/endocardial diseases; primary myocardial diseases including myocarditis; arrhythmias; and cardiac tumors.

25 In the methods of the invention for preventing or reducing the severity of ocular and other disease associated with a COX-1 or COX-1 variant, a compound can optionally be formulated together with a pharmaceutically acceptable carrier for delivery to the subject to be  
30 treated. Suitable pharmaceutically acceptable carriers are well known in the art and include, for example, aqueous or organic solvents such as physiologically

buffered saline, glycols, glycerol, oils or injectable organic esters. A pharmaceutically acceptable carrier can also contain a physiologically acceptable agent that acts, for example, to stabilize or increase solubility of  
5 a pharmaceutical composition. Such a physiologically acceptable agent can be, for example, a carbohydrate such as glucose, sucrose or dextrans; an antioxidant such as ascorbic acid or glutathione; a chelating agent; a low molecular weight polypeptide; or another stabilizer or  
10 excipient. Pharmaceutically acceptable carriers including solvents, stabilizers, solubilizers and preservatives, are well known in the art as described, for example, in Martin, Remington's Pharm. Sci., 15th Ed. (Mack Publ. Co., Easton, 1975).

15           Ophthalmic compositions can be useful in the methods of the invention for preventing or alleviating an ocular condition. An ophthalmic composition contains an ophthalmically acceptable carrier, which is any carrier that has substantially no long term or permanent  
20 detrimental effect on the eye to which it is administered. Examples of ophthalmically acceptable carriers include, without limitation, water, such as distilled or deionized water; saline; and other aqueous media. An ophthalmic composition useful in the invention  
25 can include, for example, a soluble  $\alpha$ -2/ $\alpha$ -1 selective agonist, or an  $\alpha$ -2/ $\alpha$ -1 selective agonist as a suspension in a suitable carrier.

Topical ophthalmic compositions useful for alleviating an ocular condition include, without  
30 limitation, ocular drops, ocular ointments, ocular gels and ocular creams. Such ophthalmic compositions are easy to apply and deliver the active compound effectively.

A preservative can be included, if desired, in an ophthalmic composition useful in a method of the invention. Such a preservative can be, without limitation, benzalkonium chloride, chlorobutanol, 5 thimerosal, phenylmercuric acetate, or phenylmercuric nitrate. Vehicles useful in a topical ophthalmic composition include, yet are not limited to, polyvinyl alcohol, povidone, hydroxypropyl methyl cellulose, poloxamers, carboxymethyl cellulose, hydroxyethyl 10 cellulose and purified water.

A tonicity adjustor also can be included, if desired, in an ophthalmic composition administered to alleviate an ocular condition without concomitant sedation according to a method of the invention. Such a 15 tonicity adjustor can be, without limitation, a salt such as sodium chloride, potassium chloride, mannitol or glycerin, or another pharmaceutically or ophthalmically acceptable tonicity adjustor.

Various buffers and means for adjusting pH can 20 be used to prepare an ophthalmic composition useful in the invention, provided that the resulting preparation is ophthalmically acceptable. Such buffers include, but are not limited to, acetate buffers, citrate buffers, phosphate buffers and borate buffers. It is understood 25 that acids or bases can be used to adjust the pH of the composition as needed. Ophthalmically acceptable antioxidants useful in preparing an ophthalmic composition include, yet are not limited to, sodium metabisulfite, sodium thiosulfate, acetylcysteine, 30 butylated hydroxyanisole and butylated hydroxytoluene.

Those skilled in the art can formulate a compound that modulates, differentially modulates,

specifically binds, or differentially binds a COX-1 variant to ensure proper compound distribution and bioavailability in vivo. For example, some regions of the eye can be inaccessible to some systemically administered drugs, and as a result topical drug delivery can be used. Polymers can be added to ophthalmic solutions to increase bioavailability (Ludwig and Ootenhgm, S.T.P. Pharm. Sci., 2:81-87 (1992)). In addition, colloidal systems such as, without limitation, liposomes, microparticles or nanoparticles can be used to increase penetration of a compound into the eye. Ocular drug absorption also can be enhanced using, for example, iontophoresis, prodrugs, and cyclodextrins.

Methods of ensuring appropriate distribution in vivo also can be provided by rechargeable or biodegradable devices, particularly where concentration gradients or continuous delivery is desired. Various slow release polymeric devices are known in the art for the controlled delivery of drugs, and include both biodegradable and non-degradable polymers and hydrogels. Polymeric device inserts can allow for accurate dosing, reduced systemic absorption and in some cases, better patient compliance resulting from a reduced frequency of administration. Those skilled in the art understand that the choice of the pharmaceutical formulation and the appropriate preparation of the compound will depend on the intended use and mode of administration.

A compound that modulates or specifically binds to a COX-1 variant, or that is otherwise identified by a screening method of the invention can be administered to a subject by any effective route. Suitable routes of administration include, but are not limited to, oral, topical, intraocular, intradermal, parenteral,

intranasal, intravenous, intramuscular, intraspinal, intracerebral and subcutaneous routes. The present invention also provides compounds containing an acceptable carrier such as any of the standard  
5 pharmaceutical carriers, including phosphate buffered saline solution, water and emulsions such as an oil and water emulsion, and various types of wetting agents.

A method of the invention is practiced by peripherally administering to a subject an effective  
10 amount of a compound that modulates or differentially modulates a COX-1 variant or another compound identified by a method of the invention. As used herein in reference to such a compound, the term "peripherally administering" or "peripheral administration" means  
15 introducing the compound into a subject outside of the central nervous system. Thus, peripheral administration encompasses any route of administration other than direct administration to the spine or brain.

An effective amount of a compound of the  
20 invention can be peripherally administered to a subject by any of a variety of means depending, for example, on the type of condition to be alleviated, the pharmaceutical formulation, and the history, risk factors and symptoms of the subject. Routes of peripheral  
25 administration suitable for the methods of the invention include both systemic and local administration. As non-limiting examples, an effective amount of a compound of the invention can be administered orally; parenterally; by subcutaneous pump; by dermal patch; by  
30 intravenous, intra-articular, subcutaneous or intramuscular injection; by topical drops, creams, gels or ointments; as an implanted or injected extended release formulation; or by subcutaneous minipump or other

implanted device, and by inhalation by aerosol and similar devices.

One skilled in the art understands that  
5 peripheral administration can be local or systemic. Local administration results in significantly more of a compound of the invention being delivered to and about the site of local administration than to regions distal to the site of administration. Systemic administration  
10 results in delivery of a compound of the invention essentially throughout at least the entire peripheral system of the subject.

Routes of peripheral administration useful in the methods of the invention encompass, without  
15 limitation, oral administration, topical administration, intravenous or other injection, and implanted minipumps or other extended release devices or formulations. A compound of the invention can be peripherally administered, without limitation, orally in any  
20 acceptable form such as in a tablet, pill, capsule, powder, liquid, suspension, emulsion or the like; an aerosol; as a suppository; by intravenous, intraperitoneal, intramuscular, subcutaneous or parenteral injection; by transdermal diffusion or  
25 electrophoresis; topically in any acceptable form such as in drops, creams, gels or ointments; and by minipump or other implanted extended release device or formulation. A compound of the invention optionally can be packaged in unit dosage form suitable for single administration of  
30 precise dosages, or in sustained release dosage form for continuous controlled administration.

Chronic pain and other chronic conditions such as, without limitation, chronic neurological conditions

can be alleviated using any of a variety of forms of repeated or continuous administration as necessary. In the methods of the invention for alleviating chronic pain or another chronic condition, means for repeated or  
5 continuous peripheral administration include, without limitation, repeated oral or topical administration, and administration via subcutaneous minipump. As non-limiting examples, a method of the invention can be practiced by continuous intravenous administration via  
10 implanted infusion minipump, or using an extended release formulation.

It is understood that slow-release formulations can be useful in the methods of the invention for alleviating chronic pain or other chronic conditions such  
15 as, without limitation, a chronic neurodegenerative conditions. It is further understood that the frequency and duration of dosing will be dependent, in part, on the alleviation desired and the half-life of the compound of the invention and that a variety of routes of  
20 administration are useful for delivering slow-release formulations, as detailed hereinabove.

A compound of the invention can be peripherally administered to a subject to alleviate an ocular condition by any of a variety of means depending, in  
25 part, on the characteristics of the compound to be administered and the history, risk factors and symptoms of the subject. Peripheral routes of administration suitable for alleviating an ocular condition in a method of the invention include both systemic and local  
30 administration. In particular embodiments, a pharmaceutical composition containing a compound of the invention is administered topically, or by local

injection, or is released from an intraocular or periocular implant.

Systemic and local routes of administration useful in alleviating an ocular condition according to a method of the invention encompass, without limitation, oral gavage; intravenous injection; intraperitoneal injection; intramuscular injection; subcutaneous injection; transdermal diffusion and electrophoresis; topical eye drops and ointments; periocular and intraocular injection including subconjunctival injection; extended release delivery devices such as locally implanted extended release devices; and intraocular and periocular implants including bioerodible and reservoir-based implants.

In one embodiment, a method of the invention for alleviating an ocular condition is practiced by administering an ophthalmic composition containing a compound of the invention topically to the eye. The  $\alpha$ -2/ $\alpha$ -1 selective agonist can be administered, for example, in an ophthalmic solution (ocular drops). In another embodiment, an ophthalmic composition containing a compound of the invention is injected directly into the eye. In a further embodiment, an ophthalmic composition containing a compound of the invention is released from an intraocular or periocular implant such as a bioerodible or reservoir-based implant.

As indicated above, an ophthalmic composition containing a compound of the invention can be administered locally via an intraocular or periocular implant, which can be, without limitation, bioerodible or reservoir-based. An implant refers to any material that does not significantly migrate from the insertion site

following implantation. An implant can be biodegradable, non-biodegradable, or composed of both biodegradable and non-biodegradable materials; a non-biodegradable implant can include, if desired, a refillable reservoir.

5   Implants useful in a method of the invention for alleviating an ocular condition include, for example, patches, particles, sheets, plaques, microcapsules and the like, and can be of any shape and size compatible with the selected site of insertion, which can be,  
10   without limitation, the posterior chamber, anterior chamber, suprachoroid or subconjunctiva of the eye. It is understood that an implant useful in the invention generally releases the implanted pharmaceutical composition at an effective dosage to the eye of the  
15   subject over an extended period of time. A variety of ocular implants and extended release formulations suitable for ocular release are well known in the art, as described, for example, in U.S. Patent No. 5,869,079 and 5,443,505.

20               An effective dose of a compound for use in a method of the invention can be determined, for example, by extrapolation from the concentration required in a COX-1 or COX-1 variant binding or activity assay such as one of the assays disclosed herein above. An effective  
25   dose of a compound for the treatment of a disease associated with COX-1 or a COX-1 variant also can be determined from appropriate animal models, such as transgenic mice. As non-limiting examples, animal models for pathologies such as cardiovascular disease and ocular  
30   diseases are well-known in the art. An effective dose for preventing or reducing the severity of a disease is a dose that results in either partial or complete alleviation of at least one symptom of the disease. The appropriate dose of a compound for treatment of a human

subject can be determined by those skilled in the art, and is dependent, for example, on the particular disease being treated, nature and bioactivity of the particular compound, the desired route of administration, the  
5 gender, age and health of the individual, and the number of doses and duration of treatment.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within  
10 the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

#### EXAMPLE I

##### 15 Identification of Alternatively Spliced COX-1 Variants

This example describes the molecular cloning of several alternatively spliced COX-1 variants and their expression in cell culture.

Total RNA derived from human heart, brain,  
20 lung, spleen, small intestine, skeletal muscle, kidney and liver tissue were purchased from Clontech. Total RNA was isolated from human eyes (NDRI; Philadelphia, Pennsylvania) and human ocular tissues (ciliary smooth muscles, trabecular meshwork, ODM-2) using a Qiagen total  
25 RNA isolation kit, according to the manufacturer's instructions. The ODM-2 cell line is derived from human non-pigmented ciliary epithelial cells (Escribano et al., J. Cell. Physiol. 160:511-521 (1994)). Using 5 µg of human total RNA, first strand cDNA was synthesized using  
30 SuperScript II RNase H reverse transcriptase (Life Technologies; Carlsbad, California). Reactions (20 µl) containing 5 µg of RNA, 250 ng of oligo (dT), and 100

units of reverse transcriptase were incubated at 42°C for 1 hour and terminated by incubation at 100°C for 3 minutes. The PCR buffer contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl, 2.5 units AmpliTaq DNA  
5 polymerase, 0.2 µM upstream and downstream primers, in a final volume of 50 µl. After an initial incubation for 5 minutes at 94°C, samples were subjected to 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C in a PE 9700 thermal cycler.

10               The primers used for detection of alternatively spliced COX-1 variants were as follows:

Human COX-1 Forward: GGTTCTTGCTGTTCTGCTC (SEQ ID NO: 11)  
and

15 Human COX-1 Reverse: TCACACTGGTAGCGGTCAAG (SEQ ID NO: 12)

The PCR products were isolated from a 1.5% lower melting agarose gel, and subcloned into the TOPO PCR II vector (Invitrogen; Carlsbad, California).

20 Nucleotide sequencing of the vectors was performed by Sequetech (Mountain View, California).

Full length cDNAs for COX-1 variants ALT-1 to ALT-4 were isolated and subcloned into TOPO pCDNA3.1 PCR cloning vector (Invitrogen; Carlsbad, California) or  
25 pCEP4 expression vector (Invitrogen) to create Alt COX1/pCDNA3.1 plasmids or Alt COX1/pCEP4 plasmids. Alt COX1/pCDNA3.1 plasmids were used for transient transfection, and Alt COX1/pCEP4 plasmids were used for stable transfection. Full length  $G\alpha_{16}$  cDNA was subcloned  
30 into the pCDNA3.1 vector. The plasmids were sequenced by Sequetech.

Chinese Hamster Ovary (CHO) and COS-7 cells were obtained from the American Type Culture Collection (ATCC). These cells were routinely maintained in DMEM with 10% fetal bovine serum, 1% glutamine, 0.5% penicillin/streptomycin. Cells were kept in humidified 5% CO<sub>2</sub>, 95% air at 37°C. For stable transfection, Alt COX1/pCEP4 plasmids were transfected into CHO and COS-7 cells using Fugene 6 (Roche Diagnostics Corp., Inc.; Indianapolis, Indiana), according to the manufacture's instructions, and then 200 mg/ml hygromycin was used to select cell clones that stably expressed the plasmid.

In addition, the COX-1 variants were cloned into the baculovirus expression vector pBlueBac 4.5/V5-His (Invitrogen) to create Alt COX1/pBlueBac plasmids. Baculovirus expression was performed in Sf9 or Sf21 cells obtained from Invitrogen. Briefly, Sf9 or Sf21 cells were maintained in Grace's insect media and media supplements (TC yeastolate, lactalbumin hydrolysate and L-glutamine) in humidified 5% CO<sub>2</sub>, 95% air at 25°C. Sf9 or Sf21 cells (1x10<sup>6</sup>) were infected with viral stocks at a multiplicity of infection of 3 for expression of COX-1 variants. In stable transfection, tunicamycin was added to a final concentration of 10 mg/ml to insect cells one hour after infection, and cells were cultured and harvested after 48 hours.

## EXAMPLE II

### Screening Assays Using Alternatively Spliced COX-1 Variants

This example describes assays based on enzyme activity for identifying compounds that modulate alternatively spliced COX-1 variants.

### A. Prostaglandin Synthesis Assays

After 48 hours of transfection, HEK 293/EBNA or Sf9 or Sf21 cells are preincubated with a compound for 30 minutes at 25°C. Arachidonic acid (100  $\mu$ l, final concentrations 5 or 30  $\mu$ M) is then added for an additional 10 minute incubation at 37°C. Supernatants are assayed for COX activity by radioimmunoassay (RIA) or enzyme immunoassay (Cayman Chemical Inc.) for PGE<sub>2</sub> accumulation. Assays are performed multiple times in triplicate. Inhibition curves are constructed and IC<sub>50</sub> values are determined using KaleidaGraph 3.5.

One example of the PGE<sub>2</sub> assay is as follows. Compounds (0.001-100  $\mu$ M) are preincubated with enzyme for 20 minutes in 50 mM KPO<sub>4</sub>, pH 7.5, 1  $\mu$ M heme, 0.01% phenol, 0.3 mM epinephrine. Following a 10 minute incubation of arachidonic acid, PGE<sub>2</sub> formed as a function of COX activity is detected by ELISA (Caymen, Ann Arbor, MI).

### B. *N,N,N,N'*-tetramethyl-*p*-phenylenediamine (TMPD)

#### 20 Turnover Assay

Another assay that can be used for screening compounds against alternatively spliced COX-1 variants is a substrate turn-over assay such as a TMPD turn-over assay. Half-maximal inhibition (IC<sub>50</sub>) is determined by measuring the turnover of TMPD in a spectrophotometric assay. Arachidonic acid is used as a hydroperoxide source, along with the peroxidase substrate TMPD as a co-substrate. Compounds are incubated for 1 minute with purified COX-1 or COX-1 variants in 1 mM heme, 0.1 M Tris-HCl, pH 8.1. The reaction is started by addition of 100 mM arachidonic acid, 170  $\mu$ M TMPD and measured by a

change in absorbance at 611 nm. Either the initial rate (linear for approximately 10 seconds) is measured or time points at 1 and 5 minutes are taken. Time-dependent inactivation curves are made by incubating 10 mM compound with enzyme for 5 seconds to 2 minutes. Kits for determining COX-1 activity using the TMPD turn-over assay are commercially available, for example, from Cayman Chemical Inc. (Ann Arbor, MI).

#### 10 Chemiluminescent Assay for COX-1 Peroxidase Activity

A chemiluminescent substrate can be used to detect the peroxidative activity of a COX-1 variant or other COX enzyme. After inhibition by NSAIDs, the direct residual activity of COX is measured by addition of arachidonic acid and a luminescent substrate. Light emission, directly proportional to the COX-1 variant activity in the sample, is measured over 5 seconds.

A sample protocol is as follows:

1. Prepare enzyme dilutions, buffers and Arachidonic Acid Stock Solution.
2. Pipet buffer, hematin and COX-I or COX-II and inhibitor into duplicate tubes or wells and incubate.
3. Simultaneously pipet or inject the Substrate and Arachidonic Acid into the tubes or wells.
4. Immediately read in a suitable luminometer or chemiluminescent detector for 5 seconds.
5. Calculate COX-1 variant or other COX enzyme concentration from the Standard Curve.

Kits for determining COX-1 activity using a chemiluminescent assay for COX-1 peroxidase activity are commercially available, for example, from Assay Designs,

### EXAMPLE III

This example shows the tissue distribution of alternatively spliced COX-1 variant ALT-1 to ALT-4 mRNA using RT-PCR.

PCR reactions contained the following: PCR  
20 buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl),  
2.5 units AmpliTaq DNA polymerase, 0.2  $\mu$ M forward and  
reverse primers, in a final volume of 50  $\mu$ l. After an  
initial incubation for 5 minutes at 94°C, samples were  
subjected to 30 cycles of 30 seconds at 95°C, 30 seconds  
25 at 58°C, and 30 seconds at 72°C in a PE 9700 thermal  
cycler.

	ALT-1 F	TACATTTAGGAGCCGGGATG	(SEQ ID NO: 31)
	ALT-1 R	TGGTGCTGGCATGGATAGTA	(SEQ ID NO: 32)
	ALT-2 F	TACATTTAGGAGCCGGGATG	(SEQ ID NO: 33)
	ALT-2 R	GCATCTGGCAACTGCTTCTT	(SEQ ID NO: 34)
5	ALT-3 F	GCCATGGAGTTCAACCATCT	(SEQ ID NO: 35)
	ALT-3 R	ATCTCCCGAGACTCCCTGAT	(SEQ ID NO: 36)
	ALT-4 F	TACATTTAGGAGCCGGGATG	(SEQ ID NO: 37)
	ALT-4 R	TGGTGCTGGCATGGATAGTA	(SEQ ID NO: 38)

The results of these assays are shown in

10 Figures 8 and 9. In particular, COX-1 variant ALT-1 mRNA was expressed in all of the tissue types examined. COX-1 variant ALT-2 was expressed at various levels in the tissues examined, with low to undetectable levels found in skeletal muscle. COX-1 variant ALT-3 also was

15 expressed at various levels in the tissues examined, with low to undetectable levels found in liver, lung, skeletal muscle and heart. In addition, human COX-1 variant ALT-4 mRNA was expressed at various levels in different tissues, with low to undetectable levels found in liver,

20 brain, small intestine, skeletal muscle and heart. In addition, COX-1 variant ALT-4 mRNA was present in low to undetectable levels in the neuronal cell line SK-N-SH, but was induced by 20% fetal bovine serum treatment of these cells (see Figures 9 and 10).

**EXAMPLE IV****Induction of COX-1 Variant ALT-4 in Response to Fetal Bovine Serum**

This example shows alternatively spliced COX-1  
5 variant ALT-4 mRNA is induced in SK-N-SH cells in  
response to treatment with 20% fetal bovine serum (FBS).

The neuronal cell line SK-N-SH was used in the  
following experiment. SK-N-SH cells were treated with  
20% FBS for 3 hours. At 1, 3, 6, and 24 hours  
10 post-treatment with cell culture media containing 20%  
FBS, cells were harvested and total RNA was isolated  
using the Qiagen total RNA Isolation kit. RT-PCR was  
then performed using the procedure and primers disclosed  
above for COX-1 variants ALT-1 and ALT-4.

15 As shown in Figure 10, induction of COX-1  
variant ALT-4 mRNA in SK-N-SH cells was observed by as  
three hours post induction.

All journal article, reference and patent  
citations provided herein, including referenced sequence  
20 accession numbers of nucleotide and amino acid sequences  
contained in various databases, in parentheses or  
otherwise, whether previously stated or not, are  
incorporated herein by reference in their entirety.

25 Although the invention has been described with  
reference to the disclosed embodiments, those skilled in  
the art will readily appreciate that the specific  
experiments detailed are only illustrative of the  
invention. It should be understood that various  
30 modifications can be made without departing from the  
spirit of the invention.